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(54) **Coccidiosis vaccines.**

(57) The invention provides an immunogenic polypeptide having the amino acid sequence

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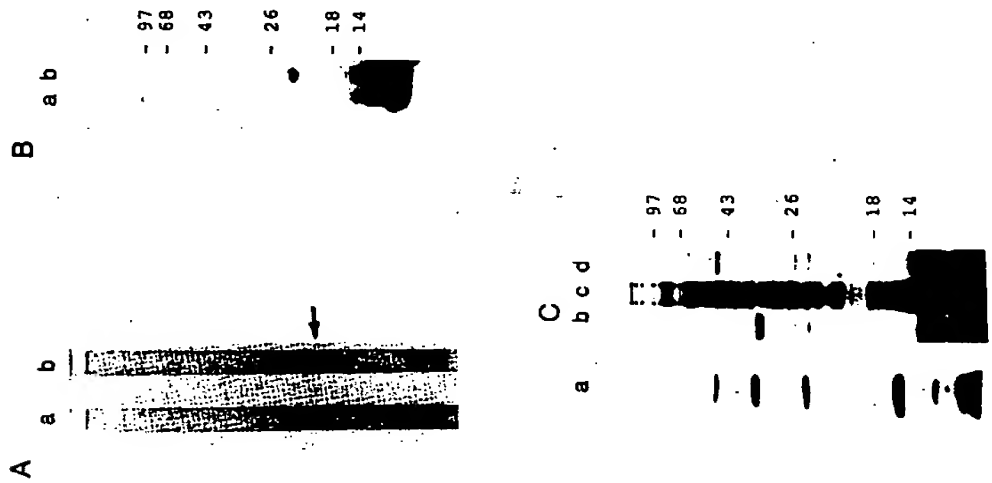
M A K S M L S G I V F A G L V A A A A A
S S A N S A A N V S V L E S G P A V Q E
V P A R T V T A R L A K P L L L L S A L
A A T L A A A F L V L Q C F N I I S S N
N Q Q T S V R R L A A G G A C G D E E D
A D E G T S Q Q A S R R R R K P D T P A
A D K Y D F V G G T P V S V T E P N V D
E V L I Q I R N K Q I F L K N P W T G Q
E E Q V L V L E R Q S E E P I L I V A R
T R Q T L E G Y L G S Q A L A Q D G K T
A K E E K V E G G K T H R R Y K V K S S
D P G Y G F P Y T T V L D G V P V G T D
E D G Y V V E V L M K T G P H G G V D M
M T S T A S Q G K F C G V L M D D G K G
N L V D G Q G R K I T A V I G M L T Q P
D T E F R S G P G D D E D D E (SEQ ID NO:1)

and fragments thereof, which polypeptides are capable of inducing an immune response against Eimeria parasites, and the DNA encoding such polypeptides, as well as recombinant vectors and recombinant viruses containing the said DNA or fragments thereof and transformed microorganisms containing such vectors and viruses and coccidiosis vaccines comprising such polypeptides. The present invention relates also to methods for producing the said polypeptides and the transformed microorganisms. The present case also relates to methods for protecting a subject against coccidiosis using the said polypeptides. The polypeptides of the invention can be administered for such protection either as purified polypeptides or in the form of DNA encoding the polypeptide in a suitable viral vector such as vaccinia virus.

FIG 2



This application relates to a novel antigen of Eimeria protozoan parasites. This antigen can be used, through various routes of administration, to protect poultry against coccidiosis.

Coccidiosis is a disease of poultry caused by intracellular protozoan parasites of the genus Eimeria. The disease is endemic in large, intensive poultry breeding establishments. The estimated cost of control of the disease through chemotherapy exceeds $100 million each year in the United States of America alone. The development of resistance to the known anti-coccidial drugs necessitates a continuing development of new agents, at a time when drug development is becoming increasingly expensive and consumer acceptance of drug residues in food animals is diminishing.

Protective immunity to natural coccidiosis infection has been well documented Controlled, daily administration of small numbers of viable oocysts for several weeks has been shown to result in complete immunity to a challenge infection of a normally virulent dose [Rose et al., Parasitology 73:25 (1976); Rose et al., Parasitology 88:199 (1984)]. The demonstration of acquired resistance to infection suggests the possibility of constructing a vaccine to induce immunity in young chickens, circumventing the need for chemical coccidiostats. In fact, such a concept has been tested in the Coccivac™ formulation of Sterwin Laboratories, Opelika, AL.

With a view to producing a coccidiosis vaccine, Murray et al., European Patent Application, Publication No. 167,443, prepared extracts from sporozoites or sporulated oocysts of Eimeria tenella which contain at least 15 polypeptides, many of which were associated with the surface of the sporozoite. Injection of these extracts into chickens reduced cecal lesions following oral inoculation with virulent E. tenella sporulated oocysts.

More recently, Schenkel et al., U.S. Patent No. 4,650,676, disclosed the production of monoclonal antibodies against E. tenella merozoites. Using these antibodies, Schenkel et al. identified a number of antigens against which the antibodies were directed. By pre-incubating E. tenella sporozoites with these antibodies and then introducing the treated sporozoites into the ceca of chickens, Schenkel et al. were able to show some reduction in cecal lesion scores, compared to untreated sporozoite controls.

Using recombinant DNA methodology, Newman et al. (European Patent Application, Publication No. 164 176) have cloned a gene from the sporozoite stage coding for a 25,000 dalton antigen from Eimeria tenella. Sera from chickens immunized by repeated immunization with killed E. tenella sporozoites immunoprecipitated this antigen from iodinated sporocyst and sporozoite membrane preparations. More recently, Jenkins [Nucleic Acids Res. 16:9863 (1988)] has described a cDNA encoding a part of a 250,000 dalton merozoite surface protein from Eimeria acervulina. The expression product of this cDNA was recognized by antiserum against the organism.

Advances in recombinant DNA technology have made another approach available, i.e. a subunit vaccine. Examples of such subunit vaccines are described e.g. in European Patent Application, Publication Nos. 324 648, 337 589 and 344 808.

The present invention provides immunogenic polypeptides having the amino acid sequence (1)(SEQ ID NO: 1)

M A K S M L S G I V F A G L V A A A A A
S S A N S A A N V S V L E S G P A V Q E
V P A R T V T A R L A K P L L L L S A L
A A T L A A A F L V L Q C F N I I S S N
N Q Q T S V R R L A A G G A C G D E E D
A D E G T S Q Q A S R R R R K P D T P A
A D K Y D F V G G T P V S V T E P N V D
E V L I Q I R N K Q I F L K N P W T G Q
E E Q V L V L E R Q S E E P I L I V A R
T R Q T L E G Y L G S Q A L A Q D G K T
A K E E K V E G G K T H R R Y K V K S S
D P G Y G F P Y T T V L D G V P V G T D
E D G Y V V E V L M K T G P H G G V D M
M T S T A S Q G K F C G V L M D D G K G
N L V D G Q G R K I T A V I G M L T Q P
D T E F R S G P G D D E D D E (SEQ ID NO: 1)

which polypeptides are capable of inducing an immune response against Eimeria parasites, for example in chickens. The Eimeria merozoite surface antigen precursor protein described herein has sequences which correspond to sequence (1)(SEQ ID NO: 1).

The preferred polypeptide of the present invention is an immunogenic polypeptide having the amino acid sequence (1)(SEQ ID NO: 1) but lacking the signal peptide sequence at the N-terminus. The present invention also relates to a functional equivalent polypeptide thereof having an amino acid sequence which is related to the said amino acid sequence by deletions, insertions or substitutions without essentially changing the immunological properties of the said polypeptide.

This invention still further provides a DNA encoding all or part of the Eimeria merozoite surface antigen precursor protein such as the DNA having the nucleotide sequence (A)(SEQ ID NO: 2)

ATGGCTAAGTCTATGCTTTCTGGAATTGTTTTTGCTGGTCTTGTTGCTGCTGCAGCG
GCCAGTTCGGCCAACAGCGCCGCCAACGTCTCCGTTTTGGAGAGTGGGCCCGCTGTG
CAGGAAGTGCCAGCGCGCACGGTCACAGCTCGCCTGGCGAAGCCTTTGCTGCTTCTT
TCTGCTCTTGCTGCGACTTTGGCAGCAGCTTTCCTCGTTTTGCAATGCTTCAACATC
ATCTCCAGCAACAACCAGCAAACCAGCGTCAGGAGACTGGCCGCCGGAGGTGCATGC
GGAGATGAGGAAGATGCAGATGAGGGAACTTCACAGCAGGCCAGCCGGAGGAGGAGA
AAACCTGATACCCCTGCAGCAGATAAATACGATTTTGTTGGCGGAACTCCAGTTTCG
GTCACTGAGCCGAATGTTGATGAAGTCCTTATCCAAATTAGAAATAAACAAATCTTT
TTGAAGAACCCATGGACTGGACAAGAAGAACAAGTTCTAGTACTGGAACGACAAAGT
GAAGAACCCATTCTGATTGTGGCGAGGACAAGACAAACACTTGAAGGATATCTTGGT
AGTCAAGCTCTTGCACAGGACGGAAAGACTGCTAAAGAAGAGAAAGTTGAAGGAGGC
AAAACTCACAGAAGATATAAAGTCAAGAGCAGCGACCCAGGATATGGATTCCCATAC
ACCACGGTGCTCGACGGGGTTCCTGTGGGAACAGACGAAGACGGATACGTCGTCGAA
GTTCTTATGAAAACCGGACCCCATGGAGGAGTCGACATGATGACTAGCACAGCATCA
CAAGGAAAATTCTGCGGAGTGCTTATGGATGACGGAAAAGGAAACCTAGTCGATGGA
CAAGGGAGAAAAATTACCGCCGTTATCGGCATGCTAACTCAACCGGATACCGAGTTT
AGAAGCGGACCAGGAGACGACGAGGACGACGAGTGA

or parts thereof such as the nucleotide sequence (B) which corresponds to the nucleotide sequence (A)-(SEQ ID NO: 2) but lacks the nucleotide sequence encoding the signal peptide sequence. An ATG codon is preferably added at the beginning of the DNA consisting of a partial sequence of the DNA having the nucleotide sequence (A)(SEQ ID NO: 2 using methods well-known in the art. The present invention still further provides recombinant vectors containing and capable of directing the expression of the said DNA in compatible host organisms, and microorganisms containing such vectors.

This invention still further provides a method for producing the polypeptides defined above, which method comprises:

(a) culturing a microorganism containing a recombinant vector comprising a DNA having a nucleotide sequence encoding the said polypeptide such as the DNA having the nucleotide sequence (A)(SEQ ID NO: 2 or a fragment thereof, such as the nucleotide sequence (B), under conditions in which the DNA sequence or fragment is expressed; and

(b) isolating the recombinant polypeptide from the culture.

This invention still further provides vaccines for the protection of subjects (e.g. human or animals) against coccidiosis comprising an effective amount of one or more of the polypeptides of the invention and a physiologically acceptable carrier. A preferred subject is fowl or poultry (e.g. chickens or turkeys). Other subjects may be domestic animals such as rabbits or sheep.

This invention still further provides vaccines for the protection of subjects against coccidiosis comprising a recombinant virus containing a DNA sequence encoding a polypeptide of the present invention, which recombinant virus is capable of causing the expression of the said DNA sequence, and a physiologically acceptable carrier.

This invention still further provides a method for the protection of subjects against coccidiosis, which method comprises administering an effective amount of a vaccine of the invention to a subject such as a young fowl which is susceptible to coccidiosis.

The Eimeria polypeptides of the present invention are important vaccine antigens because they were identified by the use of antibodies in the sera of animals that had been immunized against the coccidiosis organism and had developed immunity thereto. Because of this, it is most likely that these polypeptides play a significant role in the protection of poultry against coccidiosis.

BRIEF DESCRIPTION OF THE FIGURES

The invention can be more readily understood by reference to the figures, in which:

Fig. 1 shows the nucleotide sequence of the 1.2 kb cDNA molecule encoding the Eimeria precursor protein recognized by antigen-select antibodies from rabbit and by chicken immune sera. As can be seen from Fig. 1, the nucleotide sequence encoding the said precursor protein is contained between the ATG at nucleotide 68 and the stop codon TAA at nucleotide 1013 (coding for 315 amino acids). Fig. 1 also shows the amino acid sequence of the Eimeria precursor protein predicted from the nucleotide sequence provided. Standard single-letter abbreviations are used to represent nucleotides and amino acids. The meanings of these abbreviations can be found in standard biochemistry textbooks, such as Lehninger, Principles of Biochemistry, 1984, Worth Publishers, Inc., New York, pp. 96, 798.

Fig. 2 shows the results of an SDS PAGE analysis of various Eimeria merozoite proteins. Panel A is an immunoblot of total merozoite proteins probed with control (a) or antigen-select (b) antibodies. The arrow in Panel A indicates the position of a band containing a protein having molecular weight of about 23 kilodaltons. Panel B is an autoradiogram of ^{125}I-surface-labeled merozoite proteins immunoprecipitated with control (a) or antigen-select (b) antibodies. Panel C shows the complete mixture of products produced by the in vitro translation of merozoite polyA mRNA (c) and translation products which had been immunoprecipitated with antibodies selected using the lambda 5-7 clone (b), antibodies selected using another phage clone which produced proteins reactive with anti-merozoite serum (a) and control antibodies selected from merozoite serum using non-recombinant phage (d). The bands were visualized by fluorography. The positions of molecular weight markers having the indicated molecular weight in kilo Daltons (kDa) are shown to the right of the figure.

Fig. 3 shows the results of Southern Blot analysis of Eimeria tenella sporulated oocyst genomic DNA which has been digested with PvuII (lane 1), HincII (lane 2), PstI (lane 3), SphI (lane 4) or SacI (lane 5) using the 5-7 gene EcoR1 insert described below as probe. The positions of standard DNAs having the indicated sizes in kb are shown to the right of the figure.

Fig. 4 shows a schematic drawing of the plasmid pDS56/RBSII (not drawn to scale). In this diagram and in Figs. 6, 8 and 10, the abbreviations and symbols B, E, H, P, S, X and Xb indicate cleavage sites for restriction enzymes BamHI, EcoRI, HindIII, PstI, SalI, XhoI and XbaI, respectively.

represents the regulatable promoter/operator element N25OPSN25OP29;

represents ribosomal binding sites RBSII, RBSII(-1) or RBSII(-2) as indicated;

represents coding regions under control of these ribosomal binding sites;

represents terminators t_o or T1 as indicated;

represents the region required for DNA replication in E. coli (repl.);

represent coding regions for chloramphenicol acetyltransferase (cat) and beta-lactamase (bla), respectively.

Fig. 5 displays the complete nucleotide sequence of the plasmid pDS56/RBSII. In this sequence, the recognition sequences of the restriction enzymes depicted in Fig. 4 are indicated. The amino acid sequence shown represents the open reading frame under control of ribosomal binding site RBSII.
Fig. 6 is a schematic drawing of the plasmid pDS56/RBSII(-1) [not drawn to scale].
Fig. 7 displays the complete nucleotide sequence of plasmid pDS56/RBSII(-1). In this sequence, the recognition sequences of the restriction enzymes depicted in Fig. 6 are indicated. The amino acid sequence shown represents the open reading frame under control of ribosomal binding site RBSII(-1).
Fig. 8 is a schematic drawing of the plasmid pDS56/RBSII(-2) [not drawn to scale].
Fig. 9 displays the complete nucleotide sequence of plasmid pDS56/RBSII(-2). In this sequence, the recognition sequences of the restriction enzymes depicted in Fig. 8 are indicated. The amino acid sequence shown represents the open reading frame under control of ribosomal binding site RBSII(-2).
Fig. 10 is a schematic drawing of the plasmid pDMI.1 [not drawn to scale]. The symbols and abbreviations have the same meaning as stated in the legend to Fig. 4, but

represents coding regions for lac repressor (lacI) and neomycin phosphotransferase (neo), respectively.
Fig. 11, i.e. Figures 11A, 11B and 11C display the complete nucleotide sequence of plasmid pDMI.1. In this sequence, the recognition sequences of the restriction enzymes depicted in Fig. 10 are indicated. The amino acids shown enclose the open reading frames encoding the neomycin phosphotransferase (Met to Phe) and the lac repressor (Met to Gln; please note the reverse orientation of this gene).
Fig. 12 is a schematic drawing of the plasmid pUC8-TK-7.5K. In this diagram and in Figures 13 and 15 the abbreviation TK stands for the thymidine kinase gene sequence of vaccinia virus, 7.5K stands for the vaccinia virus 7.5K promoter, lac Z contains regulatory sequences and coding information for a part of the N-terminus of the beta-galactosidase gene, ori represents the region required for DNA replication in E. coli and AmpR stands for the coding region of the beta-lactamase gene.
Fig. 13 is a schematic drawing of the recombinant plasmid pR3.
Fig. 14 shows the complete nucleotide sequence of the recombinant plasmid pR3. The amino acid sequence shown represents the open reading frame under the control of the vaccinia 7.5K promoter.
Fig. 15 is a schematic drawing of the plasmid pR4. ML stands for the malaria leader sequence.

DESCRIPTION OF THE INVENTION

All references cited herein are hereby incorporated in their entirety by reference.

As used herein, the following terms shall have the following meanings:

"Eimeria surface antigen" means a protein having an apparent molecular weight of about 23 kilodaltons in SDS PAGE which is present in the merozoite stage of Eimeria tenella. This protein appears to be produced by post-translational processing of the in vivo expression product of a gene whose cDNA sequence is shown in Fig. 1.

"Precursor protein" means a protein having an apparent molecular weight of about 33 kilodaltons in SDS PAGE. This protein is believed to be processed by proteolysis in vivo to the Eimeria surface antigen. The nucleotide sequence of a cDNA molecule encoding the precursor protein and the amino acid sequence predicted therefrom are shown in Fig. 1.

The term "immunogenic polypeptides having the amino acid sequence (1) which polypeptides are capable of inducing an immune response against Eimeria parasites" means polypeptides capable of eliciting a B-cell and/or T-cell mediated protective immune response against Eimeria parasites comprising the said merozoite surface antigen which corresponds to sequences of the immunogenic polypeptides. The said immunogenic polypeptides may be the mature Eimeria merozoite surface antigen protein free of other Eimeria proteins per se, or fragments of the said Eimeria surface antigen protein which fragments are still capable of specifically binding to antibodies which are present in the sera of animals that are infected with an Eimeria parasite. These polypeptides correspond to T-cell and B-cell epitopes of the Eimeria surface antigen defined above. The polypeptides of the present invention may also be functional equivalents of the said Eimeria merozoite surface antigen protein, which polypeptides have an amino acid sequence related to the amino acid sequence of Fig. 1 by amino acid substitutions, which substitutions do not substantially alter the immunological activity (i.e., which do not substantially destroy the immunoreactive and/or antigenic determinants).

An example of a fragment is an immunogenic polypeptide which has the amino acid sequence (1)(SEQ

ID NO. 1) except that it lacks essentially the first 20 to about 100 amino acid residues, constituting the signal peptide sequence. Another example is an immunogenic polypeptide which has an apparent molecular weight of 23 kilodaltons on an SDS-polyacrylamide gel. Preferred fragments are as follows:

SNNQQTSV (2)(SEQ ID NO: 3)
CGDEEDADEGTSQQASRRRRKPDTPAADK (3)(SEQ ID NO: 4)
PNV (4)(SEQ ID NO: 5)
RNKQIF (5)(SEQ ID NO: 6)
NPWTGQEE (6)(SEQ ID NO: 7)
RQSEE (7)(SEQ ID NO: 8)
TRQTLE (8)(SEQ ID NO: 9)
QDGKTAKEEKVEGGKTHRRYKVKSSDPGYG (9)(SEQ ID NO: 10)
TDEDG (10)(SEQ ID NO: 11)

TGPHG (11)(SEQ ID NO: 12)
ASQGK (12)(SEQ ID NO: 13)
DDGKGNLVDGQGRK (13)(SEQ ID NO: 14) and
TQPDTEFRSGPGDDEDDE (14)(SEQ ID NO: 15).

The fragments of this invention, like the immunogenic polypeptide of sequence (1)(SEQ ID NO. 1), are capable of inducing an immune response against coccidiosis in a subject. Preferred subjects are fowl such as chickens.

Amino acid substitutions in proteins which do not substantially alter biological and immunological activities have been known to occur and have been described, e.g., by Neurath et al., in "The Proteins", Academic Press, New York (1979), in particular in Fig. 6 at page 14. The most frequently observed amino acid substitutions are Ala/Ser, Val/Ile, Asp/Glu, Thr/Ser, Ala/Gly, Ala/Thr, Ser/Asn, Ala/Val, Ser/Gly, Tyr/Phe, Ala/Pro, Lys/Arg, Asp/Asn, Leu/Ile, Leu/Val, Ala/Glu, Asp/Gly, and vice versa.

Because of the degeneracy of the genetic code, it will be understood that there are many potential nucleotide sequences (functional equivalents) that could code for the amino acid sequence (1)(SEQ ID NO. 1). It should also be understood that the nucleotide sequences of the DNA sequences and fragments of the invention inserted into vectors may include nucleotides which are not part of the actual structural genes, as long as the recombinant vectors containing such sequence or fragments are capable of directing the production in an appropriate host organism of a polypeptide of the present invention.

DNA sequences encoding the polypeptides which are functional equivalents of the said Eimeria merozoite surface antigen can readily be prepared using appropriate synthetic oligonucleotides in primer-directed site-specific mutagenesis on the exemplary cDNA of this invention (SEQ ID NO. 2), as described by Morinaga et al. [Biotechnology 2:636 (1984)].

Fragments or parts of the Eimeria merozoite surface antigen protein or the DNA encoding it can be produced by enzymatic cleavage of the larger molecules, using restriction endonucleases for the DNA and proteases for the proteins. The fragments of the invention, however, are not limited to the products of any form of enzymatic cleavage but include sub-sequences, the termini of which do not correspond to any enzymatic cleavage points. Such fragments can be made, e.g., by chemical synthesis, using the sequence data provided herein. DNA fragments can also be produced by incomplete complementary DNA (cDNA) synthesis from isolated messenger RNA (mRNA). Protein fragments can also be produced by expressing DNA fragments encoding the protein fragments. Such protein fragments can be useful in the present invention if they contain a sufficient number of amino acid residues to constitute an immunoreactive and/or antigenic determinant. Generally, at least about 7 or 8 residues are needed. As explained below, it may be necessary to couple such fragments to an immunogenic carrier molecule, to make them immunoreactive.

Immune reactivity may include both production of antibodies by B-cells (humoral immunity) and

activation of T-cells (cellular immunity). The polypeptides of the subject invention include B-cell antigenic determinants, or epitopes, and T-cell epitopes. Humoral immunity may be demonstrated by the induction of antibody production by B-cells in vivo or in vitro. Cell-mediated immunity may be demonstrated by T-cell activation, for example by increased T-cell protein synthesis, or by the stimulation of B-cells by activated T-cells. Assays for both types of immunity are well known in the art.

The polypeptides of the present invention can be made by methods known in the art such as by recombinant DNA methodology, chemical synthesis or by isolation from Eimeria preparations. When produced in accordance with this invention, the polypeptide of sequence 1(SEQ ID NO.1) and fragments thereof are substantially free of other proteins produced by Eimeria parasites.

DNA needed to make the proteins of this invention could be chemically synthesized, using the nucleotide sequence information provided in (SEQ ID NO. 2) and in the figures. Such chemical synthesis can be carried out using any of the known methods such as the phosphoramidite solid support method of Matteucci et al. [J. Am. Chem. Soc. 103:3185 (1981)].

Alternatively, cDNA can be made from Eimeria mRNA. Messenger RNA can be isolated from Eimeria merozoites using standard techniques. These mRNA samples can be used to produce double-stranded cDNA as described by Maniatis et al. [Molecular Cloning: A Laboratory Manual, 1982, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY]. This cDNA can then be inserted into an appropriate cloning vector which can be used to transform a suitable host organism (e.g. E.coli) to produce a cDNA library.

The cDNA library can then be screened using the cloned gene of this invention, or fragments thereof, as probes. Such gene or fragments can be labeled, e.g., by nick-translation using Pol I DNA polymerase in the presence of the four deoxyribonucleotides, one of which contains ^{32}P in the alpha position (Maniatis et al., supra, p. 109), for use as probes. The probes may also be prepared by oligonucleotide synthesis based on the known sequence of the cDNA of the Eimeria surface antigen.

Although Eimeria tenella was used as an mRNA source in the Examples below, the cloned genes from this species can be used as probes to isolate genes from other species of Eimeria, due to DNA sequence homology among the various species.

Once identified and isolated, the Eimeria DNAs of this invention are inserted into an appropriate expression vehicle or vector which contains the elements necessary for transcription and translation of the inserted gene sequences. Useful cloning vehicles may consist of segments of chromosomal, nonchromosomal and synthetic DNA sequences such as various known bacterial plasmids, virus DNA, such as phage DNA, combinations of plasmids and viral or phage DNA such as plasmids which have been modified to employ phage DNA or other expression control sequences, or yeast plasmids. Specific cloning vehicles which could be used include but are not limited to the pEV-vrf plasmids (pEV-vrfI, -2 and -3 which are described in Crowl et al., Gene 38:31 (1985)); SV40; adenovirus; yeast vectors; lambda gt-WES-lambda B; Charon 4A and 28; lambda-gt-2; M13-derived vectors such as pUC8, 9, 18 and 19, pBR313, 322 and 325; pAC105; pVA51; pACY177; pKH47; pACYC184; pUB110; pMB9; colE1; pSC101; pML21; RSF2124; pCR1 or RP4; fowlpox; vaccinia or a member of the herpesvirus family.

The insertion of the Eimeria genes into a cloning vector is easily accomplished when both the genes and the desired cloning vehicle have been cut with the same restriction enzyme or enzymes, since complementary DNA termini are thereby produced. If this cannot be accomplished, it may be necessary to modify the cut ends that are produced by digesting back single-stranded DNA to produce blunt ends, or by achieving the same result by filling in the single-stranded termini with an appropriate DNA polymerase. In this way, blunt-end ligation with an enzyme such as T4 DNA ligase may be carried out. Alternatively, any site desired may be produced by ligating nucleotide sequences (linkers) onto the DNA termini. Such linkers may comprise specific oligonucleotide sequences that encode restriction site recognition sequences. The cleaved vector and the Eimeria genes or fragments may also be modified by homopolymeric tailing, as described by Morrow [Methods in Enzymology 68:3 (1979)].

Many of the cloning vehicles that may be used in this invention contain one or more marker activities that may be used to select for desired transformants, such as ampicillin and tetracycline resistance in pBR322, ampicillin resistance and beta-galactosidase activity in pUC8, and ampicillin resistance in the pEV-vrf plasmids. Selection of host cells into which such vectors have been inserted is greatly simplified when the host cells otherwise lack the activities contributed by the vectors.

It should be understood that the nucleotide sequences of the Eimeria genes inserted at a selected site in a cloning vehicle may include nucleotides which are not part of the actual structural genes. Alternatively, the genes may contain only part of the complete wild-type gene. All that is required is that the gene fragments after insertion into a cloning vehicle are capable of directing the production in an appropriate host organism of a polypeptide or protein having at least one immunoreactive and/or antigenic determinant of the Eimeria surface antigen. Thus, the recombinant vectors comprising a DNA having a nucleotide sequence

encoding a protein of the present invention may be prepared by:

(a) inserting a DNA having a nucleotide sequence encoding the said protein into a vector;

(b) replicating the said vector in a microorganism; and

(c) isolating the recombinant vector from the microorganism.

The selection of an appropriate host organism is affected by a number of factors known in the art. These factors include, for example, compatibility with the chosen vector, toxicity of proteins encoded by the hybrid plasmid, ease of recovery of the desired protein, expression characteristics, biosafety and costs. A balance of these factors must be considered, and it must be understood that not all hosts will be equally effective for expression of a particular recombinant DNA molecule.

Suitable host microorganisms which can be used in this invention include but are not limited to plant, mammalian or yeast cells and bacteria such as Escherichia coli, Bacillus subtilis, Bacillus stearothermophilus and Actinomyces. Escherichia coli strain MC1061, which has been described by Casadaban et al. [J. Mol. Biol. 138:179 (1980)], can be used, or any other strain of E. coli K-12 containing the plasmid pRK248clts. Plasmid pRK248clts for use in other E. coli K-12 strains is described by Bernhard et al. [Meth. of Enzymol. 68:482 (1979)] and is also available from the American Type Culture Collection under accession No. ATCC 33766. The E. coli strain MC1061 is commercially available e.g. from CLONTECH Laboratories, Inc., Palo Alto, CA, USA and is also available from the American Type Culture Collection under accession No. ATCC 53338. Plasmids pDMI.1, pDS56/RBSII, -1 or -2 for use in E. coli strain M15 are described infra.

Transfer of the recombinant cloning vector into the host cell may be carried out in a variety of ways. Depending upon the particular vector/host cell system chosen, such transfer may be effected by transformation, transduction, transfection or electroporation. Once such a modified host cell is produced, the cell can be cultured and the protein expression product may be isolated from the culture.

Transformant clones producing the precursor protein of the Eimeria surface antigen are identified by screening with serum from animals immunized against glutaraldehyde-fixed sporozoites or merozoites of E. tenella. In the examples below, rabbit anti-merozoite serum was used for screening and characterizing the gene product. Parallel immunologic screening with immune chicken serum resulted in the independent isolation of the cDNA encoding the merozoite surface antigen precursor.

The specificity of the antisera used for immunological screening or immunoprecipitation can be increased by using a variation of the antibody select method of Hall et al. [Nature 311:379 (1984)]. In this method, which is described more fully below, antibodies that are specific for Eimeria proteins made by the clones are adsorbed out on filters.

The detection of Eimeria antigen producing clones can be achieved by the use of well known standard assay methods, including immunoprecipitation, enzyme-linked immunoassay (ELISA) and radioimmunoassay techniques which have been described in the literature [see, e.g., Kennet et al. (editors), Monoclonal Antibodies and Hybridomas: A New Dimension in Biological Analyses, 1980, Plenum Press, New York, pp. 376-384].

Recombinant vectors comprising a DNA encoding a variant polypeptide of the Eimeria surface antigen of the present invention may be prepared using methods well-known in the art, e.g. by site-specific mutagenesis.

Large amounts of the recombinant Eimeria polypeptides of the present invention may be produced by growing the transformed microorganisms obtained in this way in a fermentation broth comprising the necessary nutrients under conditions suitable for expression of the recombinant DNA. As produced in E. coli, the recombinant Eimeria polypeptides are usually present in the cytoplasm or in inclusion bodies of the bacteria. To free the proteins it is thus necessary to disrupt the outer membrane of the bacteria. This is accomplished by sonication, or by other mechanically disruptive means, such as by using a French pressure cell or Gaulin homogenizer [Charm et al., Meth. Enzymol. 22, 476-556 (1971)].

Cell disruption can also be accomplished by chemical or enzymatic means. Since divalent cations are often required for cell membrane integrity, treatment with appropriate chelating agents such as EDTA or EGTA might prove sufficiently disruptive to facilitate the leakage of the proteins from the cells. Similarly, enzymes such as lysozyme have been used to achieve the same result. That enzyme hydrolyzes the peptidoglycan backbone of the cell wall.

Osmotic shock can also be employed. Briefly, this can be accomplished by first placing the cells in a hypertonic solution which would cause them to lose water and shrink. Subsequent placement in a hypotonic "shock" solution would then lead to a rapid influx of water into the cells with an expulsion of the desired proteins.

Once freed from the cells, the Eimeria proteins may be concentrated by precipitation with salts such as sodium or ammonium sulfate, ultrafiltration or other methods well known to those skilled in the art. Further

purification could be accomplished by conventional protein purification techniques including but not limited to gel filtration, ion-exchange chromatography, preparative disc-gel or curtain electrophoresis, isoelectric focusing, low temperature organic solvent fractionation, or countercurrent distribution. Purification can also be carried out by immunoaffinity chromatography.

Specific methods for purifying Eimeria proteins from the organisms are known in the art (see e.g., Newman et al., European Patent Application, Publication No. 164 176).

The proteins of this invention or fragments thereof can also be chemically synthesized by a suitable method such as by exclusive solid phase synthesis, partial solid phase methods, fragment condensation or classical solution synthesis. Solid phase synthesis as described by Merrifield [J. Am. Chem. Soc. 85:2149 (1963)] is preferred.

Such synthesis is carried out with amino acids that are protected at the alpha-amino-terminus. Trifunctional amino acids with labile side-chains are also protected with suitable groups which will prevent a chemical reaction from occurring at that site during the assemblage of the peptide. The alpha-amino protecting group is selectively removed to allow subsequent reaction to take place at the amino-terminus. The conditions for the removal of the alpha-amino protecting group do not cause deprotection of the side-chain protecting groups.

The alpha-amino protecting groups are those known to be useful in the art of stepwise synthesis of peptides. Included are acyl type protecting groups (e.g., formyl, trifluoroacetyl, acetyl), aromatic urethane type protecting groups (e.g., benzyloxycarbonyl (Cbz) and substituted benzyloxycarbonyl), aliphatic urethane protecting groups (e.g., t-butyloxycarbonyl (Boc), isopropyloxycarbonyl, cyclohexyloxycarbonyl) and alkyl type protecting groups (e.g., benzyl, triphenylmethyl). The preferred protecting group is Boc. The side-chain protecting groups for Tyr include tetrahydropyranyl, tert.-butyl, triyl, benzyl, Cbz, 4-Br-Cbz and 2,6-dichlorobenzyl. The preferred side-chain protecting group for Tyr is 2,6-dichlorobenzyl. The side-chain protecting groups for Asp include benzyl, 2,6-dichlorobenzyl, methyl, ethyl and cyclohexyl. The preferred side-chain protecting group for Asp is cyclohexyl. The side-chain protecting groups for Thr and Ser include acetyl, benzoyl, trityl, tetrahydropyranyl, benzyl, 2,6-dichlorobenzyl and Cbz. The preferred protecting group for Thr and Ser is benzyl. The side-chain protecting groups for Arg include nitro, Tos, Cbz, adamantyloxycarbonyl or Boc. The preferred protecting group for Arg is Tos. The side-chain amino group of Lys may be protected with Cbz, 2-ClCbz, Tos or Boc. The 2-Cl-Cbz group is the preferred protecting group for Lys. The selection of the side-chain protecting group is based on the following: The side-chain protecting group remains intact during coupling and is not split off during the deprotection of the amino-terminus protecting group or during coupling conditions. The side-chain protecting group must be removable upon the completion of the synthesis of the final peptide, using reaction conditions that will not alter the target peptide.

Solid phase synthesis is usually carried out from the carboxy-terminus by coupling the alpha-amino protected (side-chain protected) amino acid to a suitable solid support. An ester linkage is formed when the attachment is made to a chloromethylated or hydroxymethyl resin and the resultant target peptide will have a free carboxyl group at the C-terminus. Alternatively, a benzhydrylamine or p-methylbenzhydrylamine resin is used in which case an amide bond is formed and the resultant target peptide will have a carboxamide group at the C-terminus. These resins are commercially available and their preparation is described by Stewart et al., "Solid Phase Peptide Synthesis" (2nd Edition, Pierce Chemical Co., Rockford, IL., 1984).

The C-terminal amino acid, Arg, protected at the side-chain with Tos and at the alpha-amino function with Boc is coupled to the benzhydrylamine resin using various activating agents including dicyclohexylcarbodiimide (DCC), N,N'-diisopropylcarbodiimide and carbonyldiimidazole. Following the attachment to the resin support the alpha-amino protecting group is removed by using trifluoroacetic acid (TFA) or HCl in dioxane at a temperature between 0° and 25°C. Dimethylsulfide is added to the TFA after the introduction of methionine (Met) to suppress possible S-alkylation. After removal of the alpha-amino protecting group, the remaining protected amino acids are coupled stepwise in the required order to obtain the desired peptide sequence.

Various activating agents can be used for the coupling reactions including DDC, N,N'-diisopropylcarbodiimide, benzotriazol-1-yl-oxy-tris(dimethylamino)-phosphonium hexafluorophosphate (BOP) and DCC-hydroxybenzotriazole (HOBt). Each protected amino acid is used in excess (>2.5 equivalents), and the couplings are usually carried out in DMF, CH_2Cl_2 or mixtures thereof. The extent of completion of the coupling reaction is monitored at each stage by the ninhydrin reaction as described by Kaiser et al. [Anal. Biochem. 34:595 (1970)]. In cases where incomplete coupling is determined the coupling reaction is repeated. The coupling reactions can be performed automatically on a Vega 250, Applied Biosystems synthesizer or other commercially available instrument. After the entire assemblage of the target peptide, the peptide-resin is deprotected with TFA/dithioethane and then cleaved with a reagent such as liquid HF for

1-2 hours at 0 ° C which cleaves the peptide from the resin and removes all side-chain protecting groups.

Side-chain to side-chain cyclization on the solid support requires the use of an orthogonal protection scheme which enables selective cleavage of the side-chain functions of the acidic amino acids (e.g., Asp) and the basic amino acids (e.g., Lys). The 9-fluorenylmethyl (OFm) protecting group for the side-chain of Asp and the 9-fluorenylmethoxycarbonyl (Fmoc) protecting group for the side-chain of Lys can be used for this purpose. In these cases the side-chain protecting groups of the Boc-protected peptide-resin are selectively removed with piperidine in DMF. Cyclization is achieved on the solid support using various activating agents including DCC, DCC/HOBt or BOP. The HF reaction is carried out on the cyclized peptide-resin as described above.

Purification and screening of the synthetic proteins can be carried out as described above for the recombinantly produced proteins.

Eimeria proteins can also be recovered from the organisms, from extracts of membrane proteins. Such methods can produce the complete, wild-type proteins. Monoclonal antibodies for this purpose can be produced as described by Köhler and Milstein [Nature 256:495 (1975)], using synthetic or natural Eimeria proteins as the antigen. These methods can be used to purify the 23 kd Eimeria surface antigen of this invention.

One or more of the polypeptides of this invention can be formulated into vaccines comprising the polypeptides and a physiologically acceptable carrier. Suitable carriers include, e.g., 0.01 to 0.1 M phosphate buffer of neutral pH or physiological saline solution.

Enhanced immunity against coccidiosis can be produced in one of two ways. First, an adjuvant or immunopotentiator can be added to the vaccine. Secondly, the proteins of the invention can be presented to a subject that is to be immunized in a larger form, either as a cross-linked complex or conjugated to a carrier molecule.

Suitable adjuvants for the vaccination of subjects include but are not limited to Adjuvant 65 (containing peanut oil, mannide monooleate and aluminum monostearate); mineral gels such as aluminum hydroxide, aluminum phosphate and alum; surfactants such as hexadecylamine, octadecylamine, lysolecithin, dimethyl-dioctadecylammonium bromide, N,N-dioctadecyl-N',N'-bis(2-hydroxymethyl) propanediamine, methoxyhexadecylglycerol and pluronic polyols; polyanions such as pyran, dextran sulfate, poly IC, polyacrylic acid and carbopol; peptides such as muramyl dipeptide, dimethylglycine and tuftsin; and oil emulsions. The proteins could also be administered following incorporation into liposomes or other microcarriers.

Incorporation into liposomes or other microcarriers provides a means by which the release of the vaccines can be sustained over a prolonged period of time. A pump such as an Alza osmotic pump could be used for the same purpose.

The immunogenicity of the polypeptides of the invention, especially the smaller fragments, can be enhanced by cross-linking or by coupling to an immunogenic carrier molecule (i.e., a macromolecule having the property of independently eliciting an immunological response in a host animal, to which the proteins and protein fragments of the invention can be covalently linked). Cross-linking or conjugation to a carrier molecule may be required because small protein fragments sometimes act as haptens (molecules which are capable of specifically binding to an antibody but incapable of eliciting antibody production, i.e., they are not immunogenic). Conjugation of such fragments to an immunogenic carrier molecule renders the fragments immunogenic through what is commonly known as the "carrier effect".

Suitable carrier molecules include, e.g., proteins and natural or synthetic polymeric compounds such as polypeptides, polysaccharides, lipopolysaccharides etc. A useful carrier is a glycoside called Quil A, which has been described by Morein et a. [Nature 308:457 (1984)]. Protein carrier molecules are especially preferred, including but not limited to mammalian serum proteins such as keyhole limpet hemocyanin, human or bovine gammaglobulin, human, bovine or rabbit serum albumin, or methylated or other derivatives of such proteins. Other protein carriers will be apparent to those skilled in the art. Preferably, but not necessarily, the protein carrier will be foreign to the host animal in which antibodies against the Eimeria proteins are to be elicited.

Covalent coupling to the carrier molecule can be carried out using methods well known in the art, the exact choice of which will be dictated by the nature of the carrier molecule used. When the immunogenic carrier molecule is a protein, the proteins or fragments of the invention can be coupled, e.g., using water soluble carbodiimides such as dicyclohexylcarbodiimide, or glutaraldehyde.

Coupling agents such as these can also be used to cross-link the proteins and fragments to themselves without the use of a separate carrier molecule. Such cross-linking into protein or protein fragment aggregates can also increase immunogenicity.

Administration of an effective amount of the vaccines of this invention can protect against coccidiosis, for example as caused by E. tenella infection or infection by other Eimeria species. Monoclonal antibodies

against the E. tenella antigens cross-react with E. acervulina and E. maxima in vitro. Preferred subjects are poultry such as chickens, but other subjects are within the scope of this invention. In accordance with this invention, any effective amount of vaccine may be used. The effective amount may be determined by routine experimentation using methods described below. An effective amount of the polypeptides and fragments of this invention that ranges from about 5 to about 50 micrograms/kg of body weight of the vaccinated subject is preferred, in particular a dose of about 25-50 µg/kg. Initial vaccinations are preferably followed by booster vaccinations given from one to several weeks later. Multiple boosters may be administered. The dosages of such boosters generally range from about 5 to 50 µg/kg, preferably about 20-50 µg/kg. Standard routes of administration can be used such as subcutaneous, intradermal, intramuscular, oral, anal or in ovo administration (direct injection into embryos). Single or multiple booster vaccinations may be followed by an induced minor coccidiosis infection, which can enhance protection.

The presentation of the coccidial antigens of the invention to the immune systems of subjects, for example fowl, can also be achieved by cloning genes coding for the antigens into bacteria (e.g., E. coli or Salmonella) or into viruses (e.g., poxviruses or herpesviruses) and administering the live vector system or, when appropriate, its inactivated form to the subjects, orally, by injection or by other commonly used routes. Carbit et al. [in: Vaccines, 1987, Cold Spring Harbor Laboratory, pp. 68-71] have described the use of E. coli, while Clements [Pathol. Immunopathol. Res. 6:137 (1987)] has described the use of Salmonella. Moss et al. [Ann. Rev. Immunol. 5:305 (1987)] have reviewed the use of viral vector systems employing recombinant poxviruses.

One kind of poxvirus, vaccinia virus, can be used to test the delivery of coccidial antigens in cell culture and in animals. Fowlpox virus is another poxvirus carrier that can be used for performing the present invention. For analytical studies, vaccinia virus has been found to be more practical than fowlpox virus. This is because vaccinia virus multiplies more rapidly than the avian virus and has a host range that is not restricted to avian cells. Large amounts of heterologous DNA can be inserted into the vaccinia viral genome without inhibiting viral maturation and infectivity [Smith et al., Gene 25:21 (1983)]. Multiple heterologous genes inserted into the virus are expressed in infected animals and elicit antibody production [Perkus et al., Science 229:981 (1985)].

The techniques used to produce recombinant vaccinia viruses can be readily adapted by routine procedures to fowlpox or herpesvirus systems. A recombinant virus comprising a DNA having a nucleotide sequence encoding a protein of the present invention may be prepared by:

(a) inserting a DNA having a nucleotide sequence encoding the said protein into the genome of a virus without inhibiting viral maturation and infectivity;

(b) amplifying the said recombinant virus in a cell culture; and

(c) purifying the recombinant virus from the culture.

The use of recombinant viruses as carriers in vaccines against coccidiosis is especially advantageous in that vaccinated fowl develop immunity against both the coccidial antigen and the viral carrier (i.e., such vaccines are bivalent). The utility of such vaccines can be further enhanced by inserting additional genes into the carrier virus. For example, parts of the Newcastle disease viral genome can be inserted together with a coccidial antigen gene into a fowlpox virus, thereby conferring immunity against Newcastle disease, coccidiosis and fowlpox, all with a single vaccine.

The administration of the live vector vaccines of the invention can be carried out by numerous methods well known in the art. For example, the "stick" method commonly used to vaccinate poultry against fowlpox virus can be used. This method consists of sticking or pricking the skin of the wing web with a sharp needle dipped into the vaccine. The needle usually has an eye near the tip like a sewing machine needle which carries a drop of vaccine. Alternatively, the live vaccines can be injected subcutaneously or intradermally into the wing web or any other site.

The recombinant live vector vaccines can also be added to drinking water or even sprayed over subjects, such as chicks, that are to be vaccinated. They can also be administered in feed, preferably after protective encapsulation [Balancou et al., Nature 322:373 (1986)], or in ovo. In the latter method, the viral vaccines are injected directly into embryos, in particular, chicken embryos. [Sharma, Avian Dis. 25:1155 (1985)].

Unless otherwise specified, percentages given below for solids in solid mixtures, liquids in liquids, and solids in liquids are on a wt/wt, vol/vol and wt/vol basis, respectively. Furthermore, unless otherwise specified, the suppliers of reagents and instruments mentioned below are not meant to be mandatory. The skilled person is in a position to select similar reagents or instruments from other suppliers.

EXAMPLE 1

Purification of Merozoites

Merozoites of E. tenella were harvested from the ceca of 50 infected chickens (3 week old Hubbard Cross; Avian Services, Frenchtown, NJ, USA) 5 days after infection with 50,000 of the above sporulated oocysts/bird. Similar chickens from other sources may be used. The ceca were removed and washed with phosphate buffered saline (PBS) for 15 minutes on a magnetic stirrer. The epithelial debris was partially removed by low speed centrifugation (50 x g), and the crude merozoites were recovered by centrifugation at 2,000 x g at 4°C for 10 minutes. The pellet was resuspended in Lysing Buffer (8.29 g/l NH_4Cl, 0.372 g/l Na_2EDTA, 1.0 g/l $KHCO_3$, pH 7.6) and incubated on ice for 30 minutes. The merozoites were collected by centrifugation, washed once in PBS and passed over a column containing 1.0 g of spun nylon fiber (Scrub Nylon Fiber, Fenwall Laboratories, Deerfield, IL) in a separatory funnel. The merozoites were collected by centrifugation as before and frozen on dry ice for RNA isolation, or further purified in diethylaminoethyl cellulose (DEAE, Whatman DE52, Whatman Bio Systems, Inc., Clifton, NJ, USA) for Western blot analysis.

For purification in DEAE cellulose, approximately 1 x 10^9 merozoites were applied in PBS to a 10-ml bed volume column and eluted with PBS. The merozoites were recovered in the first 100 ml of flow-through, essentially free of red blood cells and other cellular debris.

Immunoprecipitation of ^{125}I-Labeled Surface Proteins

The surface proteins of purified merozoites were labeled with ^{125}I by the IODOGEN™ method (Pierce Chemical Co.) or by use of IODOBEADS™ (Pierce Chemical Co.). For the latter procedure, 4 IODOBEADS™ were washed 3 times with 0.2 M sodium phosphate, pH 7.5, and 1-3 mCi of ^{125}I-Na were added and incubated for 5 minutes at room temperature. Purified merozoites (3 x 10^8) in 200 ml of PBS, pH 7.0, were added to the reaction vial, and the incubation was continued for 15 minutes. At the end of the incubation, phenylmethanesulfonyl fluoride (PMSF) was added to a final concentration of 5 mM.

The labeled merozoites were recovered from the incubation mixture by centrifugation at 12,000 x g for 30 seconds and solubilized in 1 ml of either 2% sodium dodecysulfate (SDS) or 1% Triton X-100 in PBS, pH 7.0. Insoluble material was removed by centrifugation for 3 minutes at 12,000 x g. The solubilized proteins were dialyzed against 3 liters of PBS, pH 7.0, at 4°C using a 3,500 molecular weight cutoff membrane to remove any residual free ^{125}I. The ^{125}I-labeled proteins (typically about 1.5 x 10^8 cpm incorporated into protein) were stored at 4°C until used. The TCA precipitable radioactivity was typically in excess of 95% of the total radioactivity.

Rabbit antiserum against glutaraldehyde-fixed merozoites was prepared as follows:

Approximately 1 x 10^9 purified merozoites were suspended in 1% glutaraldehyde in PBS and incubated at room temperature for 5 minutes. The fixed parasites were harvested by centrifugation at 2000 x g for 5 minutes, washed three times with PBS and resuspended in 1 ml PBS. New Zealand white rabbits were given multiple intradermal injections in the skin of the back with a total of 0.5 ml of the fixed parasite solution emulsified with 0.5 ml complete Freund's adjuvant. Rabbits received two booster injections containing the same parasite protein in incomplete Freund's adjuvant at two week intervals. Blood was harvested from the ear vein two weeks after the last boost and serum containing antibodies was obtained by centrifugation of coagulated blood samples for 15 minutes at 2500 x g.

Samples of labeled proteins for immunoprecipitation (5 ml, containing 5 x 10^5 cpm) were diluted into 100 ml of IP buffer (0.25% NP-40, 20 mM Tris-HCl, pH 7.5,0.15 M NaCl), pre-cleared by incubation for 20 minutes on ice with 5 mg of Staph-A protein (Pansorbin™, Calbiochem Corp., San Diego, CA), and incubated for several hours at 4°C with 5-10 ml of the rabbit anti-merozoite serum. The antibody complexes were collected by a second incubation with 5 mg of Staph-A protein for 20 minutes on ice and centrifuged for 15 seconds in an Eppendorf centrifuge. The pellets were washed 4 times with IP buffer, and the labeled proteins immunoprecipitated by the antibody reagent were eluted from the complex by heating to 100°C for 5 minutes in SDS gel sample buffer (65 mM Tris pH 6.8, 0.5% SDS, 5% beta-mercaptoethanol, 10% glycerol, 0.1% bromophenol blue). SDS polyacrylamide gelelectrophoresis (SDS PAGE) was carried out as described by Laemmli [Nature 227:680 (1970)].

Results obtained with the rabbit antiserum were confirmed using immune chicken serum prepared as follows:

Chickens were immunized by repeated infection with viable sporulated oocysts of E tenella (100,000 oocysts, given 3 times at 2 week intervals). Blood was harvested by cardiac puncture and the serum containing antibodies was separated from coagulated debris following centrifugation at 2500 x g for 5 minutes.

Comparison studies were carried out in which both the anti-merozoite rabbit serum and the immune

chicken serum were used to immunoprecipitate ^{125}I-surface-labeled Eimeria merozoite proteins and the in vitro products of the translation of poly(A)-containing merozoite RNA. The precipitated proteins were then subjected to SDS PAGE and visualized by fluorography using standard fluorography techniques and reagents.

These studies showed that many proteins from both sources were precipitated by both sera. Thus, either serum could be used to screen genetic recombinants expressing Eimeria proteins. For convenience, the rabbit anti-merozoite serum was used first in the screening procedures described below. However, immune chicken serum was used in parallel screening of the cDNA library as described below. This was essential for the identification of proteins likely to be important in the immune response to the infectious organism, because only the chicken serum was produced in response to challenge with live organisms. Only the immunized chickens were demonstrably resistant to such organisms.

To increase the specificity of the rabbit anti-merozoite serum for Eimeria proteins, antibody select was carried out on the sera essentially as described by Hall et al. [Nature 311:379 (1984)]. Briefly, antibodies specific for the precursor protein expressed by a recombinant phage clone (see below) were purified from the rabbit anti-merozoite serum as follows.

The positive phage was plated to high density and grown at 42°C for 3.5 hours. Expression of the fusion protein was induced by overlayering the plate with a nitrocellulose filter saturated with 10 mM isopropylthiogalactoside (IPTG), and incubation was continued at 37°C for 6-8 hours. The antigen-loaded filters were washed in TBS (20 mM Tris-HCl, pH 8.0,150 mM NaCl) and incubated for 8-10 hours at 4°C with excess anti-merozoite serum which had been pre-absorbed with the E. coli host bacteria. The filters were washed 3 times with TBS to remove non-specific antibodies.

The antibodies specifically bound to the fusion protein on the filters were eluted with 2.0 ml of 0.1 M glycine, pH 2.6, 0.15 M NaCl (15 minutes at 20°C). The eluted antibodies were neutralized immediately with an equal volume 0.1 M Tris-HCl, pH 8.0. The selected antibodies (hereinafter referred to as "antigen-select antibodies") were then used in the immunoprecipitation of surface-labeled merozoites or in vitro translation products, or as probes in Western blots of whole merozoite protein. Control sera were prepared using non-recombinant phage in the antigen-select procedure.

The results of Western blot and immunoprecipitation analyses using the antigen-select antibodies are shown in Fig. 2. The products of the immunoprecipitation of labeled proteins were visualized by fluorography as described by Bonner et al. [Eur. J. Biochem, 46:83 (1974)]. Numbers to the right of the figure show the positions of molecular weight marker proteins having the indicated sizes in kilodaltons.

Panel A of Fig. 2 shows an immunoblot of total merozoite proteins probed with control (a) or antigen-select antibodies (b). Panel B shows ^{125}I-surface-labeled merozoite proteins that had been immunoprecipitated with control (a), or antigen-select (b) antibodies.

Isolation and In vitro Translation of Merozoite mRNA

Frozen merozoite pellets containing 1 x 10^9 to 1 x 10^{10} organisms were thawed into 10 ml of TEL/SDS buffer (0.2 M Tris-HCl, 0.1 M LiCl, 25 mM EDTA, 1% (w/v) sodium dodecyl sulfate (SDS), pH 8.8) containing 1 mM dithiothreitol (DTT) and 300 units of RNasin (Promega Biotec, Madison, WI) and homogenized with 10-12 strokes in a teflon-coated tissue homogenizer. Insoluble debris was separated by centrifugation in the cold at 3,000 x g. The supernatant fluid was extracted twice with phenol:chloroform:isoamyl alcohol (24:24:1,v/v) which had been equilibrated with the TEL buffer.

The aqueous phase was digested with 100 mg/ml proteinase K at 37°C for 30 minutes and reextracted with an equal volume of phenol:chloroform (1:1), and the nucleic acid was precipitated with two volumes of ethanol for 1 hour on dry ice, or overnight at -20°C. The pellet, after centrifugation at 10,000 x g for one hour, was resuspended in TE (10 mM Tris-HCl, pH 7.5,2 mM EDTA) and spun through a 4 ml CsCl cushion (5.7 M CsCl, 0.1 M EDTA) at 150,000 x g for 20 hours at 15°C. The RNA pellet was reprecipitated from 0.2 M potassium acetate with 2.5 volumes of ethanol. This total RNA was passed once over oligo-dT cellulose to enrich for poly(A)$^+$ RNA, as described by Maniatis, supra, page 197. A typical yield of 1.9 mg of total RNA from 5 x 10^9 merozoites contained approximately 20 µg of poly(A)$^+$ RNA.

Between 0.1 and 0.5 µg of mRNA was used to program in vitro protein synthesis in a nuclease-treated rabbit reticulocyte lysate (Amersham Corp., Arlington Heigths, IL, USA or Promega Biotec) supplemented with 10-20 mCi of ^{35}S-methionine per 20 ml of reaction mixture. The in vitro translation products were analyzed by immunoprecipitation followed by SDS PAGE and visualized by fluorography as described above, with the results shown in Fig. 2, Panel C.

Lane c of Panel C shows the complete mixture of products programmed by the poly (A)-containing merozoite RNA. Lane b, a and d show translation products immunoprecipitated by antibodies selected by a

recombinant phage clone designated lambda 5-7 (see below; this clone expresses a gene encoding the 33 kilodalton Eimeria precursor protein), another phage clone reacting with anti-merozoite serum and a non-recombinant lambda gt1 1 clone, respectively.

It should be noted that a major protein having an apparent molecular weight of about 33 kilodaltons can be seen in lanes a and b, Figure 2, Panel C. This protein is not present in the lane containing total merozoite proteins probed with antigen-select antibodies (Panel A, lane b), but a 23 kilodalton band can be seen in this gel (Panel A, lane b, arrow). A protein of 23 kilodaltons was also immunoprecipitated by the antigen-select antibodies from ^{125}I-labelled merozoite proteins as shown in Figure 2, panel B, lane b. These observations together suggest that the 33 kilodalton precursor protein may be processed by proteolytic cleavage in mature merozoites to the 23 kilodalton surface antigen.

Preparation of a Merozoite cDNA Expression Library

Double-stranded cDNA was synthesized from 6 µg of the merozoite poly (A)$^+$ RNA as described by Gubler et al., Gene 25:263 (1983), using reverse transcriptase (BRL, Gaithersburg, MD, USA) to elongate from an oligo(dT) primer and RNase H (BRL) and E. coli DNA polymerase I (New England Biolabs, Beverly, MA, USA) to synthesize the complementary strand. The double-stranded cDNA was then blunt-ended with T4 DNA polymerase (BRL), and Eco RI linkers (GGAATTCC, Collaborative Research Inc., Bedford, MA, USA) were added after treatment with EcoRI methylase (New England Biolabs), following the manufacturers' protocols.

Following digestion with EcoRI, the cDNAs were fractionated in Biogel A-50M to remove excess linker molecules and cDNAs smaller than approximately 300 bp, as described by Huynh et al., infra. The cDNA was then concentrated by precipitation from ethanol.

A library was prepared in λgt11 (Stratagene Cloning Systems, San Diego, CA) as described by Huynh et al., in D. Glover (ed.), DNA Cloning Vol. I: A Practical Approach, 1985, IRL Press, Washington, D.C., USA, pp. 49-78. The EcoRI cDNA fragments were ligated to EcoRI digested, dephosphorylated λgt11 arms (Stratagene Cloning Systems), and the resulting DNA was packaged into phage with the Gigapack™ kit (Stratagene Cloning Systems), following the manufacturer's protocol.

The resulting library was amplified by plating on Y1088 host cells. The percentage of recombinants was estimated from the ratio of blue to colorless plaques on X-gal plates (Maniatis, supra, page 24) in the presence of isopropyl thiogalactoside (IPTG, Sigma Chemical Co.) to be about 90%.

Immunological Screening of the cDNA Library

The λgt11 merozoite cDNA expression library was plated on Y1090 cells at a density of about 10,000 plaques per 150 mm plate. Six such plates were incubated for 3.5 hours at 42°C, overlayered with nitrocellulose filters previously soaked in 10 mM IPTG to induce the expression of the beta-galactosidase fusion protein, and incubated for an additional 4-5 hours to overnight at 37°C. The filters were removed from the plates and subjected to several batchwise washes with TBS (20 mM Tris HCl, pH 8.0,0.15 M NaCl). Nonspecific protein binding sites were blocked by incubation in 20% fetal calf serum (FCS) in TBS for one hour at room temperature.

The filters were then incubated for one hour with rabbit anti-merozoite serum which had been preadsorbed with the Y1090 cells, at 1:100 dilution in TBS containing 20% calf serum. Nonspecific antibodies were removed in successive washes with TBS, one of which contained 0.1% NP-40. The filters were incubated with goat anti-rabbit peroxidase conjugate (BioRad, Richmond, CA) at 1:1000 dilution in TBS plus calf serum for one hour at room temperature. The color reaction was developed with 4-chloro-1-naphthol (BioRad) following the manufacturer's instructions.

Serum from immune chicks was also used for the screening. This serum was preadsorbed with Y1090 cells and used at the same dilution as the rabbit serum. Rabbit anti-chicken antibody was used as the secondary antibody, and goat anti-rabbit horseradish peroxidase conjugate was used as the detecting antibody. Single plaques were isolated in a secondary screen using the same reagents.

One clone, designated lambda 5-7, produced a protein that was strongly reactive with antibodies from the rabbit serum. A second isolate, 1-5 was identified by screening with immune chick serum, and proved to contain a cDNA insert of the same size as the 5-7 clone. The DNA sequence analysis indicated that these phage clones encoded the same merozoite antigen.

Expression of the Lambda 5-7 cDNA in E. coli

A 1.2 kb insert from lambda 5-7 was isolated by EcoRI digestion and agarose gel electrophoresis [Maniatis et a., supra, pp. 157-170]. The EcoRI ends were repaired with Klenow polymerase in the presence of dATP and dTTP, and BamHI linkers (GGGATCCC) were ligated to both ends. The modified fragment was inserted into each of the three expression vectors pDS56/RBSII, pDS56/RBSII,-1 and pDS56/RBSII,-2 at the BamHI site. These three vectors are described below. Plasmids containing the inserts in both possible orientations were transformed as described by Mandel et al. [J. Mol. Biol. 53:159 (1970)] into E. coli strain M15 carrying the compatible plasmid pDMI.1. The E. coli strain M15 harboring plasmids pDS56/RBSII and pDMI.1 is described in European Patent Application, Publication No. 316 695.

Plasmid Construction

Generally, plasmids pDS56/RBSII, -1 and -2 contain the regulatable promoter/operator element N25OPSN25OP29 and the ribosomal binding sites RBSII, RBSII(-1) and RBSII(-2), respectively. These ribosomal binding sites were derived from the ribosomal binding site of the promoter P_{G25} of the E. coli phage T5 [European Patent Application, Publication No. 207 459] and were obtained via DNA synthesis.

Due to the high efficiency of expression, the above-mentioned plasmids can be maintained in E. coli cells only if the promoter/operator element is repressed by the binding of a lac repressor to the operator. The lac repressor is coded in the lacI gene. N25OPSN25OP29 can be repressed efficiently only when a sufficient number of repressor molecules is present in the cells. Therefore, the $lacI^q$ allele, which contains a promoter mutant responsible for an increased expression of the repressor gene, was used. This $lacI^q$ allele is present on the plasmid pDMI.1, as described below.

The pDMI.1 plasmid carries, in addition to the lac I gene, the neomycin phosphotransferase gene, which confers kanamycin resistance to the bacteria and which is used as the selection marker. pDMI.1 is compatible with the pDS56/RBSII, -1 and -2 plasmids. E. coli cells which are transformed with expression vectors pDS56/RBSII, -1 and -2 must contain pDMI.1 to guarantee that the expression vector is held stable in the cells. Induction of this system is achieved by adding IPTG to the medium.

Plasmid pDS56/RBSII

The part of pDS56/RBSII which lies between the restriction cleavage sites for XbaI and XhoI and which contains the replication region and the gene for beta-lactamase (which confers ampicillin resistance to the cells) (Figs. 4 and 5) was derived originally from the plasmid pBR322 [Bolivar et al., Gene 2:95-113 (1977); Sutcliffe, Cold Spring Harbor Symp. Quant. Biol. 43: 77-90 (1979)]. However, the gene for beta-lactamase is modified by elimination of the cleavage sites for the restriction enzymes HincII and PstI. These alterations in the DNA sequence have no effect on the amino acid sequence of the beta-lactamase. The remaining part of the plasmid carries the regulatable promoter/operator element N25OPSOP29 followed by the ribosomal binding site RBSII, which is part of an EcoRI/BamHI fragment, cleavage sites for the restriction enzymes SalI, PstI and HindIII, the terminator to of E. coli phage lambda [Schwarz et al., Nature 272: 410-414 (1978)], the promoter-free gene of chloramphenicol acetyltransferase [Marcoli et al., FEBS Letters, 110: 11-14 (1980)] and the terminator T1 of the E. coli rrnB operon [Brosius et al., J. Mol. Biol. 148: 107-127 (1981)].

Plasmid pDS56/RBSII(-1)

Plasmid pDS56/RBSII(-1) (Figs. 6 and 7) is similar to plasmid pDS56/RBSII but contains the ribosomal binding site RBSII(-1).

Plasmid pDS56/RBSII(-2)

Plasmid pDS56/RBSII(-2) (Figs. 8 and 9) is similar to plasmid pDS56/RBSII but contains the ribosomal binding site RBSII(-2).

The difference in these three plasmids is that they differ by one nucleotide following the ATG start codon resulting in protein expression from all three potential reading frames.

Plasmid pDMI.1

Plasmid pDMI.1 (Figs. 10 and 11) carries the gene for neomycin phosphotransferase from the transposon Tn5 [Beck et al., Gene 19: 327-336 (1982)], which confers kanamycin resistance to E. coli cells, and the lacI gene [Farabough, Nature 274: 765-769 (1978)) with the promoter mutation I^q [Calos, Nature

274: 762-765 (1978)], which codes for the lac repressor. Moreover, plasmid pDMI.1 contains a region of the plasmid pACYC184 [Chang and Cohen, J. Bacteriol. 134: 1141-1156 (1978)], which contains all information required for the replication and stable transmission to the daughter cells.

It should be understood that in addition to the above-described plasmid, any E. coli expression system is contemplated to be useful in this experiment.

The bacterial transformants were grown at 37°C in LB medium [Maniatis et al., supra, page 68] and expression of protein induced by addition of 1 mM IPTG to the medium. After incubating for 1 hour, 1-ml samples were taken, and the cells in the samples were collected by centrifugation. The cell pellets were treated as described by Crowl et al., supra, and the lysates were subjected to SDS PAGE. Following electrophoresis, the proteins in the gels were either stained with Coomassie brilliant blue or transferred to nitrocellulose membranes for Western blot analysis [Towbin et al., Proc. Nat. Acad. Sci. USA 76:4350 (1979); Burnetti, Anal. Biochem. 112:195 (1981)], using the rabbit anti-merozoite serum as described above.

The analysis showed that the 1.2 kb cDNA molecule encoded in one orientation in all three reading frames a protein that migrated with an apparent molecular weight of about 33 kilodaltons as measured by SDS-PAGE and reacted with the antibodies from the rabbit anti-merozoite serum.

DNA Sequence Analysis

In general, small scale isolation of plasmid DNA from 1 ml of saturated overnight cultures were carried out using the procedure of Birnboim et al. [Nucleic Acids Research 7: 1513 (1979)]. This procedure allows the isolation of a small quantity of DNA from a bacterial colony for analytical purposes. Larger amounts of plasmid DNA were prepared using 1-liter cultures following a standard protocol with cesium chloride gradient centrifugation [Maniatis et al., supra, page 93].

The DNA sequence of the 1.2 kb EcoRI cDNA insert from lambda 5-7 was determined as follows. The insert was digested with EcoRI, purified by gel electrophoresis, and ligated to the EcoRI digested pEV-vrf plasmid described by Crowl et al. [Gene 38: 31 (1985)]. This plasmid was designated pEV/5-7 and was used to propagate the 1.2 kb cDNA insert for hybridization analysis (as described below) and in preliminary DNA sequence analysis by the method of Zagursky et al. [Gene Anal. Tech. 2: 89 (1983)].

To determine the complete DNA sequence, the 1.2 kb cDNA insert from pEV/5-7 was further subcloned into the M13 mp19 single-stranded phage vector using the BIO-RAD™ M13 Cloning Kit and the SEQUENASE™ sequencing Kit. The sequence was determined by the dideoxy chain termination method of Sanger et al. [Proc. Natl. Acad. Sci. USA 74: 5463 (1977)] following the recommended protocols in the SEQUENASE™ Kit (United States Biochemical Corp., Cleveland OH, USA).

The complete nucleotide sequence of the 1.2 kb cDNA from pEV/5-7 including 5, and 3' untranslated regions is shown in Fgure 1.

The cDNA sequence predicts an open reading frame extending from the ATG at position 68 to the TGA stop codon at position 1013 encoding 315 amino acid residues as shown in Figure 1.

The theoretical size of 33,375 Dalton for this protein correlates with the immunoprecipitated product from the in vitro translation of merozoite mRNA (see Figure 2, panel C, lane a) using the antigen-select reagent and with the protein expressed from the cDNA in the E. coli expression vectors described above.

Analysis of the deduced amino acid sequence of the protein encoded by the lambda 5-7 cDNA insert (Fig. 1) shows that depending on the algorithmus used for the prediction the first 20 or 75 to 95 amino-terminal amino acid residues have a overall hydrophobic character, suggestive of a possible signal peptide function. The signal peptide sequence may therefore consist of up to about the first one hundered amino-terminal amino acid residues. This in view of the fact that it has been found that the polypeptide obtained after in vitro translation of merozoite mRNA and purified by immunoprecipitation shows a molecular weight of about 35 kDa in its precursor form and about 23 kDa in its mature form. As mentioned above the size of the precursor form is in good agreement with the theoretical size of the protein. However, the mature form may also represent an internal or N-terminal fragment of the precursor molecule. The exact amino terminus may be determined by known methods.

For a number of regions of the polypeptide, with the stated amino acid sequence, epitopes can be designated based on a combination of the hydrophilicity criteria according to J.P. Hopp and K.R. Woods [Proc.Natl.Acad.Sci.USA 78: 3824-3828 (1981)] and secondary structure criteria according to P.Y. Chou and G.D. Fasman [Advances in Enzymology 47: 45-148 (1987)].

The following regions contain probable epitopes for antibodies:

S_{79} - V_{86} (SEQ ID NO: 3)

C_{95} - K_{123} (SEQ ID NO: 4)

P_{137} - V_{139} (SEQ ID NO: 5)

R_{147} - F_{152} (SEQ ID NO: 6)
N_{155} - E_{162} (SEQ ID NO: 7)
R_{169} - E_{173} (SEQ ID NO: 8)
T_{181} - E_{186} (SEQ ID NO: 9)
Q_{196} - G_{225} (SEQ ID NO: 10)
T_{239} - G_{243} (SEQ ID NO: 11)
T_{252} - G_{256} (SEQ ID NO: 12)
A_{265} - K_{269} (SEQ ID NO: 13)
D_{276} - K_{289} (SEQ ID NO: 14)
T_{298} - E_{315} (SEQ ID NO: 15)

In addition, T-cell epitopes may be derived on theoretical grounds according to Berzofsky's amphiphilicity criterion [Good et al. Science 235, 1059-1062 (1987)]. T-cell epitopes are processed from antigens [H.M. Grey and R. Chestnut, Immunol. Today 6:101-106 (1985), transported intracellularly [Schwartz A.L., Ann.Rev.Immun.8: 195-229 (1990)], and recognized by the T-cell receptor complex. Although some of the algorithms were designed primarily to identify Class II antigenic sites, because of the similarities with Class I peptide interactions, they also appear to be useful for the identification of peptide targets for cytotoxic T lymphocytes [Feller and de la Cruz, Nature 349: 720-721 (1991)]

In addition a potential glycosylation site is located at the amino acid asparagine at position 20 (D_{20}). The carbohydrate groups are known to confer important physical properties such as conformational stability, resistance to proteases, charge and waterbinding capacity. It has to be noted however that D_{20} is part of the leader sequence and may therefore not be present in the mature protein. For a review on the important role of carbohydrate groups in biological recognition see J.C. Paulson, TIBS 14: 272-276 (1989).

Hybridization Analysis

DNA was isolated from excysted, sporulated oocysts following treating with trypsin and bile and washing with PBS as follows:

The parasite material (approximately 1 x 10^9 oocysts) was suspended in 20 ml of 0.5 M EDTA, pH 8.0,0.5% sarcosyl (Sigma, St. Louis, MO, USA) and digested with proteinase K (Boehringer-Mannheim, FRG) at 0.1 mg/ml for 2 hours at 50°C, with RNase (10 mg/ml) for 1 hour at 37°C, and again with proteinase K for 1 hour at 50°C. The protein was removed with 2 extractions with phenol saturated with 20 mM Tris HCl, pH 7.5,1 mM EDTA (TE), and one extraction with phenol/chloroform (1:1). The aqueous phase was dialysed extensively against TE and concentrated by ethanol precipitation. A typical yield of 0.4 mg DNA per 1 x 10^6 oocysts was obtained.

The parasite DNA was digested with various restriction endonucleases following the manufacturers' protocols and the resulting DNA fragments were resolved by electrophoresis at 40 V for 2.5 hours in 0.8% agarose in Loening Buffer (4.7 g NaH_2PO_4, 4.36 g Tris base, 0.372 g Na_2EDTA per liter, pH 7.6). The gel was treated with 0.25 M HCl for 30 minutes, and transferred to a Zeta-Probe membrane (BIO-RAD™) in 0.4 M NaOH overnight. The filter was neutralized in 2 X SSC (pH 6.8) and baked for one hour at 80°C under vacuum.

The filter was prehybridized for 3 hours at 65°C in 7% SDS, 1% BSA (Boehringer, fraction V), 0.5 M $NaHPO_4$ buffer, pH 7.2. The 5-7 gene EcoRI insert was gel isolated following digestion of the pEV/5-7 plasmid, as described above, with EcoRI, and labeled by random-priming with Klenow fragment in the presence of ^{32}P-labeled deoxynucleotides. The labelled insert was separated from unincorporated nucleotides in Spin-Columns (BIO-RAD™), denatured and added to the hybridization solution. Following incubation for 12 hours at 65°C, the filters were washed 3 times with 2 X SSC/0.1% SDS, and twice with 0.1 X SSC/0.1% SDS at 65°C. The genomic DNA fragments hybridizing to the probe were detected by autoradiography Although the pEV/5-7 plasmid was used here, it is understood that any equivalent vector containing the 1.2 kb cDNA insert of the merozoite 5-7 gene would also perform in an acceptable manner.

The results of this analysis are shown in Fig. 3, where the results of digestion by PvuII (1), HincII (2), PstI (3), SphI (4) or Sac (5) can be seen.

Genomic DNA fragments of 6.5 and 3.6 kb were detected following digestion with PvuII and SacI, in lanes 1 and 5, respectively. Since there are no sites for these enzymes in the cDNA clone, the maximum size of the Eimeria gene can be estimated to be 3.6 kb.

Three fragments were detected following digestion with PstI (lane 3). Two PstI sites are predicted from the cDNA sequence, which would produce an internal fragment of 306 bp (too small to be detected in this Southern blot) and two joint fragments. The appearance of a third large PstI fragment is best explained by the presence of an intron located between the internal PstI sites.

The pattern of fragments produced by SphI (lane 4), which also cuts twice in the cDNA, provides no definitive information. The small internal SphI fragment of 604 bp predicted from the cDNA sequence could not have been detected in this gel.

Digestion of genomic DNA with EcoRI produced a 1.2 kb genomic fragment corresponding in size to the cDNA fragment. Double digestion with HincII and EcoRI produced a 0.9 kb fragment (not shown).

In a Northern blot analysis [Alwine et al., Proc. Natl. Acad. Sci. USA 74: 5350 (1977)] of poly(A)-containing mRNA isolated from merozoites, the 1.2 kb cDNA fragment of the lambda 5-7 gene hybridized to a single mRNA species of approximately 1.3 kb in length. From the size correlation, it is apparent that the 5-7 clone, together with the 5' extension determined from the 1-5 isolate mentioned above, represents the full-length sequence of the cDNA, with the possible exception of the extreme 5' nucleotides.

EXAMPLE 2

In order to produce a more effective way of immunizing chicks with the E. tenella merozoite antigen 5-7, the 1.2 kb cDNA described above was cloned into vaccinia virus. The recombinant vaccinia virus obtained in this way was used as a subunit coccidiosis vaccine to vaccinate chicks.

Construction of the Vector

All forms of recombinant vaccinia virus (rVV) made were based on homologous recombination into the viral thymidine kinase (TK) locus as described by Macket et. a. [Proc.Natl.Acad.Sci.USA 79: 7415 (1982)]. The TK locus has been mapped to the vaccinia virus (VV) HindIII J fragment [Hruby et al., J. Virol. 43: 403 (1982)], and part of this fragment has been sequenced [Weir et al., J. Virol. 46:530 (1983)].

The construction of the vector for recombination pUC8-TK-7.5K is basically described in European Patent Application, Publication No. 344 808. Briefly, the vector consists of a pUC8 plasmid backbone carrying the vaccinia virus TK gene disrupted by the VV 7.5K promoter [Venkatesan et al., Cell 25: 805 (1981)]. Downstream of the promoter in the direction of the transcription, a multiple cloning site has been constructed to allow the introduction of the gene to be expressed. Fig.12 shows the schematic drawing of the pUC8-TK-7.5K plasmid.

To generate our construct the EcoRI fragment encoding the merozoite 5-7 gene was cloned into the EcoRI site of the polylinker contained in the basic vector shown in Fig.12. Constructs containing the fragment in the correct orientation were propagated and modified as described below to delete an inframe start codon situated 97 nucleotides upstream of the natural start codon of the merozoite 5-7 gene. For this purpose the plasmid was digested with the restriction enzymes SmaI and BglII. After rendering the BglII site blunt with Klenow enzyme in the presence of the four deoxyribonucleotides, the plasmid was religated and the construct pR3 (Fig.13), carrying the expected deletion, was propagated and used for recombination into the vaccinia virus. Fig.14 shows the complete sequence of this recombination plasmid.

In addition the merozoite 5-7 gene was also introduced into the vector for recombination containing the DraI-HindIII malarial antigen leader. This vector, which is described in European Patent Application, Publication No. 344 808, is based on vector pUC8-TK-7.5K but has in addition, downstream of the 7.5K promoter, the sequence of the 190 kDa malaria antigen leader. Adjacent to this leader sequence a multiple cloning site has been constructed to allow the introduction of the gene to be expressed. The resulting transcript driven by the VV 7.5K promoter will be translated into a protein, carrying at the N-terminus the 190 kDa malaria antigen leader. In vivo processing at the potential cleavage site of the leader sequence leads to the mature protein. Fig.15 shows the schematic drawing of this construct pR4 carrying the merozoite 5-7 gene.

Construction of Recombinant Vaccinia Virus

CV1 cells plated on a 8 cm^2 culture plate were adapted to 33° C and grown to 80-90% confluency were infected with 0.1 plaque forming units (pfu) per cell of the vaccinia virus temperature sensitive mutant ts N7 [Drillien, R. and Spehner, D. Virology 131: 385-393 (1983)]. After 2 hours at the permissive temperature of 33° C in a CO_2 incubator [Kieny et al., Nature 312: 163 (1984)] the cells were transfected with 0.25 ml of a calcium phosphate DNA precipitate as described in Weir et al., [Proc.Natl.Acad.Sci.USA 79: 1210-1214 (1982)]. The calcium-phosphate-DNA precipitate mixture consisted of: 0.8% NaCl, 0.038% KCl, 0.0134 M $Na_2HPO_4 \cdot 2H_2O$, 0.1% Glucose; pH 7.0 and 125 mM $CaCl_2$, 200 ng of the vaccinia wild type DNA (WR strain) and 100 ng of the appropriate recombinant plasmid pR3 or pR4. After one hour at room temperature additional medium was added to the plate followed by an incubation for 2 hours at 39.5° C in a 5% CO_2

incubator. At this temperature, the ts N7 virus cannot replicate, resulting in a selection for viruses which have recombined at least in the ts 7 locus.

After two days of incubation at 39.5° C the cells were harvested by scraping and the suspension was further disrupted by sonication. This homogenate was then used to obtain TK negative (TK^-) virus by titration on human TK^- 143 cells in the presence of 30 µg/ml of bromodeoxyuridine (BUdR). Plaques were picked and the virus was further plaque-purified two more times in human TK^- 143 cells in the presence of 30 µg/ml of BUdR. Virus stocks were then made in CV1 cells in the absence of BUdR. The recombinant vaccinia virus R3.2 and R4.1 respectively were checked for the presence of the 1.2 kb cDNA merozoite gene inserted into the TK gene, by digesting the viral DNA with HindIII and comparing the rVV DNA pattern to a pattern of wild type (WR) vaccinia DNA digested with the same restriction enzyme. If recombination had occurred a shift in the HindIII J DNA fragment should be seen after electrophoresis in a 1% agarose gel. This was indeed observed. The shift correlated with the calculated values deducted from the insert size.

Test for Expression

To test for the expression of the merozoite 5-7 antigen by the recombinant virus, CV1 cells were infected with either rVV R3.2 or rVV R4.1 and harvested 48 hours later. After centrifugation of the cells, the pellets were solubilized in Laemmli sample buffer [Nature 227: 680 (1970)] in the presence of beta-mercaptoethanol as a reducing agent. After boiling for 5 minutes, the samples were loaded on a 12.5% SDS-PAGE slab gel. After electrophoretic separation the proteins were blotted onto nitrocellulose membranes (Trans-Blot, BIO RAD) in Blot-buffer: 25 mM Tris-HCl, 0.19 M glycine; pH 8.3 and 20% (v/v) methanol at 80 V constant voltage in a Transblot transfer cell (BIO RAD Laboratories) for 2 hours at 4° C [Towbin et a., Proc. Natl. Acad. Sci.USA 76: 4350-4354 (1979)].

For immuno-detection the nitrocellulose was pretreated with 5% non-fat milk powder in TBS (20 mM Tris-HCl, 150 mM NaCl, adjusted to pH 8) for 45 minutes and incubated for 2 hours or overnight at room temperature with a 1:50 dilution of the rabbit anti-E. tenella merozoite serum in TBS buffer containing 20% (v/v) fetal calf serum. The nitrocellulose was then washed three times for 10 minutes in TBS containing 0.1% NP40 before incubation for 2 hours at room temperature with an affinity-purified goat-anti-rabbit IgG (H + L) peroxidase conjugate (BIO-RAD) at a 1:1000 dilution in TBS and 5% non-fat milk powder (H + L stands for heavy and light chaines of IgG). The blots were then washed three times as described above. Binding of the peroxidase conjugate was detected by reacting the nitrocellulose in 0.018% 4-chloro-1-naphthol in 6% methanol in H_2O and 0.02% (v/v) H_2O_2. The reaction was stopped by washing the blot extensively in H_2O.

It was found that the rabbit anti E.tenella merozoite serum reacted in Western blots (Towbin et al; supra) with CV1 cells, infected with the rVV R3.2, with two distinct protein bands of 33 kDa and 23 kDa, respectively. BIO-RAD prestained SDS-PAGE molecular weight standards (low range) were used as a reference. CV1 cells infected with wild type WR vaccinia virus did not react with the rabbit anti E. tenella merozoite serum. The size of the 33 kDa protein correlates with the theoretically expected value of the precursor protein as shown in Fig. 2, panel C, lane b). The smaller 23 kDa protein could be the processed version of the precursor protein mentioned above, which was also seen on the surface of the merozoite (see Fig. 2, panel A and B, lane b). The same results were observed with CV1 cells infected with the rVV R4.1.

In addition it could further be shown by Western blot analysis that immune serum from chickens infected with sporulated E. tenella oocysts recognized the merozoite 5-7 proteins (33 kDa and 23 kDa) expressed in CV1 cells infected with the vaccinia virus recombinants R3.2 and R4.1, respectively

Production of Virus

The WR strain virus can multiply in almost all cell types [Drillien et al., J. Virology 28: 843 (1978)], and its multiplication can be observed directly through formation of plaques. In most cases we used CV1 cells to prepare large stocks of the virus.

For infection, the cell culture medium was removed from 80-90% confluent CV1 cells growing in 175 cm^2 culture flasks (e.g. Falcon 3028), and the cells were incubated in a PBS solution containing virus (0.1 pfu/ml, 0.01 ml/cm^2) for one hour at room temperature (20 • C). Fresh cell culture medium was then added (0.2 ml/cm^2), and the flasks were incubated at 37°C for 2-3 days until about 80% of the cells had lysed. The resulting stock solution was stored directly with cells and medium in the original culture flasks at - 30°C before virus purification.

The following purification steps were used to obtain a virus preparation free of host cell specific components. Infected cell cultures which had been stored at -30°C were thawed and the remaining cells

were freed from the surface of the flasks by shaking or scraping. The cells and viruses were centrifuged out of the medium (Sorvall centrifuge GSA rotor, one hour at 5000 rpm, 10°C). The pellet of cells with the virus particles was resuspended in PBS (10-20 x the volume of the pellet) and centrifuged as above. This pellet was then resuspended in a 10-fold volume of RSB Buffer (10 mM Tris-HCl adjusted to pH 8.0,10 mM KCl, 1 mM $MgCl_2$).

To lyse the remaining intact cells and free the virus from the cell membranes, the above suspension was subjected to sonication (twice, 10 seconds at 60 watts at room temperature in a sonifier, e.g. Labsonic 1510 with a 4 mm probe). The mixture was centrifuged in a Sorval GSA rotor for 3 minutes at 3000 rpm, 10°C. A virus suspension, free from cell nuclei and large cell debris, was thus produced. The supernatant was carefully removed, and the pellet was resuspended in RSB buffer, sonicated and centrifuged as above.

The second supernatant was combined with the first, layered onto a 10 ml 35% sucrose cushion (in 10 mM Tris-HCl pH 8.0) and centrifuged for 90 minutes at 14000 rpm in a Beckman SW 27 rotor at 10° C. The supernatant was decanted and the pellet of virus particles was resuspended in 10 ml of 10 mM Tris-HCl, pH 8.0, sonicated to homogenize the mixture (2 times for 10 seconds at room temperature as described above) and loaded onto a step gradient for further purification.

The step gradient consisted of 5 ml aliquots of sucrose in 10 mM Tris-HCl pH 8.0, of the following concentrations: 20%, 25%, 30%, 35% and 40%. This gradient was centrifuged in a Beckman SW27 rotor for 35 minutes at 14000 rpm, 10°C. Several bands containing virus particles were visible in the 30%-40% sucrose region. This region of the gradient was removed and diluted with PBS and the virus particles were sedimented (Beckmann SW27 rotor for 90 minutes at 14000 rpm at 10°C). The pellet containing almost exclusively virus particles was resuspended in PBS so that the virus concentration was on the average 0.5 - 1 x 10^{10} pfu/ml. This virus stock was used either directly or diluted with PBS.

To determine the virus concentration and the purity of the virus stock, two methods were used. The absolute concentration of virus particles was conveniently obtained by measuring the optical density (OD) of the stock solution in a spectrophotometer at the wavelength 260 nm (OD/260 nm), where 1 OD/260 equals about 1.2 x 10^{10} particles per ml [Joklik, Virology 18: 9 (1962)]. Virus concentration was also obtained by titrating the virus on cells (plaque assay), assuming that only one out of 60 virus particles can infect a cell.

To titer the virus concentration on cultured cells, chick embryo fibroblasts (CEF) cells were grown in cell culture medium on 8 cm^2 culture plates (Falcon 3001). After the cells reached 80%-90% confluency, the medium was removed, replaced with 0.2 ml of a diluted virus solution in PBS, and left at room temperature for one hour. The virus stock solution was diluted in 10-fold steps. Two ml of semi-solid cell culture medium comprising 10% agarose were added to each plate and the plates were then placed for 16-24 hours in a CO_2 incubator at 37°C. Subsequently, 2 ml of semi-solid cell culture medium containing 0.2% neutral red was layered on to stain the living cells, and the plates were incubated for an additional 16-24 hours. The colorless plaques were then counted under a microscope.

EXAMPLE 3

Chick Immunization

To determine whether the vaccinia viral vector rVV-R3.2, harboring the gene merozoite 5-7 could protect chicks against challenge by sporulated oocysts of a pathogenic strain of E. tenella, the following vaccinations were carried out.

Cockerels of the layer breed WARREN, supplied by the hatchery E. Wuethrich in Belp (Switzerland), were kept in wire-floored cages on a commercial broiler type grower diet, consisting predominantly of maize, wheat and soya-bean. On day 17, the chicks were inoculated with 3 x 10^8 pfu of the recombinant vaccinia virus R3.2 in 100 µl of PBS, whereas 50 µl were injected subcutaneously into the wing web, another 50 µl were given intramuscularly into the breast. With intervals of one week each, this procedure was repeated twice, but for one treatment group the last virus injection was replaced by an oral inoculation with 5000 sporulated oocysts of a virulent strain of E. tenella (e.g. strain T7-776/21), in order to simulate the natural exposure of chicks to infectious coccidia under field conditions. One week after the last immunization all chicks were bled for analytical purposes and another week later (day 45) the birds were challenged with 50000 sporulated oocysts of E.tenella (e.g. strain T7-776/21). The parasites were allowed to conclude their developmental cycle of 7 days and on day 52 the chicks were bled, sacrificed and necropsied. The infected ceca were removed, the lesions due to the parasitic development were scored and the whole tissue was homogenized to determine its oocyst content. Moreover, the performance of the chicks (daily weight gain and feed conversion) was recorded.

Protection Experiment

The data in Table 1 shows clearly that the vaccination with the recombinant vaccinia virus R3.2 had a considerable protective effect against the severe coccidial challenge. The lesion scores were reduced by 18% and the oocyst content in the ceca by 35% in comparison to the infected control. Performance, which is economically the most important parameter, was improved by 62% for the daily weight gain and by 56% for the feed conversion. However, when the last virus injection was replaced by a mild coccidiosis infection, the protection of chicks against coccidiosis was nearly complete. Performance of these chicks was equivalent to the non-infected controls and lesion scores as well as oocyst content of the ceca were low. Since a single inoculation with E. tenella has never been demonstrated to confer such a high degree of protection, it was concluded that the virus based vaccination had strongly primed the immune system of the chicks so that the subsequent mild coccidial infection could exhibit a booster effect, resulting in such an effective immune protection against coccidiosis.

Humoral status of the chickens

The humoral status of the chickens was analyzed by indirect ELISA and Western blot using the following method.

Table 1

Performance and parasitological parameters of chicks, vaccinated with recombinant vaccinia virus rVV-R3.2 after challenge with 50'000 oocysts of E. tenella.

Treatment				Number of Groups pro Chicks	Mean Body Weight (g) on Day 45	Mean Body Weight (g) on Day 52	Daily Weight Gain (g) Day 45-52	Mean Feed Conversion Day 45-52	Cecal Lesion Score	Oocyst Content of the Ceca (Mio/g of Ceca)
1. Immunization *)	2. Immunization *)	3. Immunization *)	Challenge							
-	-	-	-	3/12	788.2	985.8	28.2	2.66	0	0
V	V	V	+	3/12	778.1	860.4	11.8	5.19	1.58	1.73
V	V	C	+	3/12	779.3	960.6	25.9	2.98	0.75	0.18
-	-	-	+	3/12	783.0	834.3	7.3	11.88	1.92	2.67

*) V = Injection with 3 x 10^8 PFU of recombinant virus in 100 µl per chick,
C = Inoculation with 5000 E. tenella oocysts per chick

The Western blot analysis was done as described above, except that one additional incubation step before the chicken sera samples was performed. This step consisted in incubation of the blotted nitrocellulose with hyperimmune rabbit anti vaccinia serum (2 hours at room temperature, 1:50 dilution) to cover all vaccinia specific proteins.

For the indirect ELISA, third generation merozoites of E. tenella harvested from in vitro primary chicken

kidney cell cultures were used. The microtiter plates were coated with 3000 merozoites per well dissolved in 100 ml of sodium carbonate-bicarbonate buffer (pH 9.6) and incubated overnight at 4°C. The plates were washed three times with deionized water and refilled with 200 ml of blocking buffer (0.1 M Na_2HPO_4 adjusted to pH 6.5 with HCl, 1% BSA, 0.5 g/l sodium ethylmercurithiosalicylate). The blocking lasted overnight at 4°C. Two serum samples per animal were tested. One sample was taken one week after the last immunization the second just before the animals were sacrificed. The samples were diluted in two fold steps in PBS containing 3% milk powder starting with a 1 to 50 fold dilution. Incubation was done for 4 hours at 37°C (100 ml). The plate was washed as described above followed by addition of 100 ml per well of the conjugate goat anti-chicken (H + L) labeled with peroxidase (1: 2000 dilution in PBS containing 3% milk powder). Incubation was done at 37°C for 2 hours. Antibody-antigen complexes were visualized by adding 100 ml of TMB-substrate. The TMB-substrate consisted of one part of TMB (0.24 g tetramethylbenzidin dissolved in 5 ml acetone and brought up to 50 ml with methanol) and 20 parts of substrate (0.2 M citric acid pH 4.0,275 ml/l of H_2O_2 30%).

The reaction was stopped with 0.5 M H_2SO_4 before the plates were read in a ELISA reader (Titertek Multiskan MCC/340, Flow Laboratories) at 450 nm.

After the vaccination of the chickens with the recombinant viruses R3.2 and R4.1 only a weak humoral antibody response to the coccidia merozoites (average titer 1:100), compared to the control animals (average titer >1:1600) immunized with low doses of sporulated oocysts was observed. This suggests that the positive protection and performance data of the vaccinated chickens (see Table 1 and results discussed above) may result from a cell-mediated effector mechanism.

In order to confirm the presence of specific antibodies against the merozoite 5-7 antigen, blood samples with the highest antibody titer in the ELISA were tested on Western blot, using CV1 cells infected with the rVV 3.2 as antigen source. All sera recognized two proteins of 33 kDa and 23 kDa respectively showing that a succesfull immunization had taken place.

Many modifications and variations of this invention may be made without departing from its spirit and scope, as will become apparent to those skilled in the art. The specific embodiments described herein are offered by way of example only, and the invention is to be limited only by the terms of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
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(ii) TITLE OF INVENTION: Coccidiosis Vaccines

(iii) NUMBER OF SEQUENCES: 15

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(v) CURRENT APPLICATION DATA:
APPLICATION NUMBER:
(vi) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: US 07/729,099
(B) FILING DATE: 12-JUL-1991

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 315 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella
(D) DEVELOPMENTAL STAGE: Merozoite

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Met Ala Lys Ser Met Leu Ser Gly Ile Val Phe Ala Gly Leu Val Ala
1               5                  10                  15
```

```
Ala Ala Ala Ala Ser Ser Ala Asn Ser Ala Ala Asn Val Ser Val Leu
            20                  25                  30

Glu Ser Gly Pro Ala Val Gln Glu Val Pro Ala Arg Thr Val Thr Ala
        35                  40                  45

Arg Leu Ala Lys Pro Leu Leu Leu Leu Ser Ala Leu Ala Ala Thr Leu
    50                  55                  60

Ala Ala Ala Phe Leu Val Leu Gln Cys Phe Asn Ile Ile Ser Ser Asn
65                  70                  75                  80

Asn Gln Gln Thr Ser Val Arg Arg Leu Ala Ala Gly Gly Ala Cys Gly
                85                  90                  95

Asp Glu Glu Asp Ala Asp Glu Gly Thr Ser Gln Gln Ala Ser Arg Arg
            100                 105                 110

Arg Arg Lys Pro Asp Thr Pro Ala Ala Asp Lys Tyr Asp Phe Val Gly
        115                 120                 125

Gly Thr Pro Val Ser Val Thr Glu Pro Asn Val Asp Glu Val Leu Ile
    130                 135                 140

Gln Ile Arg Asn Lys Gln Ile Phe Leu Lys Asn Pro Trp Thr Gly Gln
145                 150                 155                 160

Glu Glu Gln Val Leu Val Leu Glu Arg Gln Ser Glu Glu Pro Ile Leu
                165                 170                 175

Ile Val Ala Arg Thr Arg Gln Thr Leu Glu Gly Tyr Leu Gly Ser Gln
            180                 185                 190

Ala Leu Ala Gln Asp Gly Lys Thr Ala Lys Glu Glu Lys Val Glu Gly
        195                 200                 205

Gly Lys Thr His Arg Arg Tyr Lys Val Lys Ser Ser Asp Pro Gly Tyr
    210                 215                 220

Gly Phe Pro Tyr Thr Thr Val Leu Asp Gly Val Pro Val Gly Thr Asp
225                 230                 235                 240

Glu Asp Gly Tyr Val Val Glu Val Leu Met Lys Thr Gly Pro His Gly
                245                 250                 255

Gly Val Asp Met Met Thr Ser Thr Ala Ser Gln Gly Lys Phe Cys Gly
            260                 265                 270

Val Leu Met Asp Asp Gly Lys Gly Asn Leu Val Asp Gly Gln Gly Arg
        275                 280                 285

Lys Ile Thr Ala Val Ile Gly Met Leu Thr Gln Pro Asp Thr Glu Phe
    290                 295                 300
```

Arg Ser Gly Pro Gly Asp Asp Glu Asp Asp Glu
305 310 315

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 948 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGGCTAAGT CTATGCTTTC TGGAATTGTT TTTGCTGGTC TTGTTGCTGC TGCAGCGGCC 60

AGTTCGGCCA ACAGCGCCGC CAACGTCTCC GTTTTGGAGA GTGGGCCCGC TGTGCAGGAA 120

GTGCCAGCGC GCACGGTCAC AGCTCGCCTG GCGAAGCCTT TGCTGCTTCT TTCTGCTCTT 180

GCTGCGACTT TGGCAGCAGC TTTCCTCGTT TTGCAATGCT TCAACATCAT CTCCAGCAAC 240

AACCAGCAAA CCAGCGTCAG GAGACTGGCC GCCGGAGGTG CATGCGGAGA TGAGGAAGAT 300

GCAGATGAGG GAACTTCACA GCAGGCCAGC CGGAGGAGGA GAAAACCTGA TACCCCTGCA 360

GCAGATAAAT ACGATTTTGT TGGCGGAACT CCAGTTTCGG TCACTGAGCC GAATGTTGAT 420

GAAGTCCTTA TCCAAATTAG AAATAAACAA ATCTTTTTGA AGAACCCATG GACTGGACAA 480

GAAGAACAAG TTCTAGTACT GGAACGACAA AGTGAAGAAC CCATTCTGAT TGTGGCGAGG 540

ACAAGACAAA CACTTGAAGG ATATCTTGGT AGTCAAGCTC TTGCACAGGA CGGAAAGACT 600

GCTAAAGAAG AGAAAGTTGA AGGAGGCAAA ACTCACAGAA GATATAAAGT CAAGAGCAGC 660

GACCCAGGAT ATGGATTCCC ATACACCACG GTGCTCGACG GGGTTCCTGT GGGAACAGAC 720

GAAGACGGAT ACGTCGTCGA AGTTCTTATG AAAACCGGAC CCCATGGAGG AGTCGACATG 780

ATGACTAGCA CAGCATCACA AGGAAAATTC TGCGGAGTGC TTATGGATGA CGGAAAAGGA 840

AACCTAGTCG ATGGACAAGG GAGAAAAATT ACCGCCGTTA TCGGCATGCT AACTCAACCG 900

GATACCGAGT TTAGAAGCGG ACCAGGAGAC GACGAGGACG ACGAGTGA 948

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ser Asn Asn Gln Gln Thr Ser Val
1 5

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 29 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Cys Gly Asp Glu Glu Asp Ala Asp Glu Gly Thr Ser Gln Gln Ala Ser
1 5 10 15

Arg Arg Arg Arg Lys Pro Asp Thr Pro Ala Ala Asp Lys
20 25

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 3 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Pro Asn Val
1

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Arg Asn Lys Gln Ile Phe
1 5

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

Asn Pro Trp Thr Gly Gln Glu Glu
1 5

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Arg Gln Ser Glu Glu
1 5

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 6 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Thr Arg Gln Thr Leu Glu
1 5

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Gln Asp Gly Lys Thr Ala Lys Glu Glu Lys Val Glu Gly Gly Lys Thr
1 5 10 15

His Arg Arg Tyr Lys Val Lys Ser Ser Asp Pro Gly Tyr Gly
20 25 30

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Thr Asp Glu Asp Gly
1 5

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Thr Gly Pro His Gly
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ala Ser Gln Gly Lys
1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 14 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Asp Asp Gly Lys Gly Asn Leu Val Asp Gly Gln Gly Arg Lys
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: YES

(v) FRAGMENT TYPE: C-terminal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Eimeria tenella

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Thr Gln Pro Asp Thr Glu Phe Arg Ser Gly Pro Gly Asp Asp Glu Asp
1               5                  10                  15

Asp Glu
```

Claims

1. An immunogenic polypeptide having the amino acid sequence

```
M A K S M L S G I V F A G L V A A A A A
S S A N S A A N V S V L E S G P A V Q E
V P A R T V T A R L A K P L L L L S A L
A A T L A A A F L V L Q C F N I I S S N
N Q Q T S V R R L A A G G A C G D E E D
A D E G T S Q Q A S R R R R K P D T P A
A D K Y D F V G G T P V S V T E P N V D
E V L I Q I R N K Q I F L K N P W T G Q
E E Q V L V L E R Q S E E P I L I V A R
T R Q T L E G Y L G S Q A L A Q D G K T
A K E E K V E G G K T H R R Y K V K S S
D P G Y G F P Y T T V L D G V P V G T D
E D G Y V V E V L M K T G P H G G V D M
M T S T A S Q G K F C G V L M D D G K G
N L V D G Q G R K I T A V I G M L T Q P
D T E F R S G P G D D E D D E      (1)
```

(SEQID NO.1)

or fragments thereof which fragments are capable of inducing an immune response against Eimeria parasites, said polypeptide and fragments thereof being substantially free of other proteins produced by Eimeria parasites.

2. An immunogenic polypeptide which is a fragment of the immunogenic polypeptide of claim 1 lacking

the signal peptide sequence of said polypeptide.

3. An immunogenic polypeptide which is a fragment of the immunogenic polypeptide of claim 1 and has an apparent molecular weight of 23 kilodaltons as measured by SDS-PAGE.

4. An immunogenic polypeptide according to claim 1 which is capable of inducing a T-cell mediated immune response.

5. An immunogenic polypeptide according to claim 1 which is a peptide selected from the group of peptides comprising the amino acid sequence

SNNQQTSV (2) (SEQ ID NO. 3),
CGDEEDADEGTSQQASRRRRKPDTPAADK (3)(SEQ ID NO. 4).
PNV (4)(SEQ ID NO: 5),
RNKQIF (5)(SEQ ID NO: 6),
NPWTGQEE (6)(SEQ ID NO: 7),
RQSEE (7)(SEQ ID NO: 8),
TRQTLE (8)(SEQ ID NO: 9),
QDGKTAKEEKVEGGKTHRRYKVKSSDPGYG (9)(SEQ ID NO: 10),
TDEDG (10)(SEQ ID NO: 11),
TGPHG (11)(SEQ ID NO: 12),
ASQGK (12)(SEQ ID NO: 13),
DDGKGNLVDGQGRK (13)(SEQ ID NO: 14), or
TQPDTEFRSGPGDDEDDE (14)(SEQ ID NO: 15).

6. An isolated DNA molecule encoding an immunogenic polypeptide having the sequence of (SEQ ID NO. 1) or a partial sequence thereof which polypeptide is capable of inducing an immune response against Eimeria parasites.

7. An isolated DNA molecule having all or part of the nucleotide sequence

ATGGCTAAGTCTATGCTTTCTGGAATTGTTTTTGCTGGTCTTGTTGCTGCTGCAGCG
GCCAGTTCGGCCAACAGCGCCGCCAACGTCTCCGTTTTGGAGAGTGGGCCCGCTGTG
CAGGAAGTGCCAGCGCGCACGGTCACAGCTCGCCTGGCGAAGCCTTTGCTGCTTCTT
TCTGCTCTTGCTGCGACTTTGGCAGCAGCTTTCCTCGTTTTGCAATGCTTCAACATC
ATCTCCAGCAACAACCAGCAAACCAGCGTCAGGAGACTGGCCGCCGGALGTGCATGC
GGAGATGAGGAAGATGCAGATGAGGGAACTTCACAGCAGGCCAGCCGGAGGAGGAGA
AAACCTGATACCCCTGCAGCAGATAAATACGATTTTGTTGGCGGAACTCCAGTTTCG
GTCACTGAGCCGAATGTTGATGAAGTCCTTATCCAAATTAGAAATAAACAAATCTTT
TTGAAGAACCCATGGACTGGACAAGAAGAACAAGTTCTAGTACTGGAACGACAAAGT
GAAGAACCCATTCTGATTGTGGCGAGGACAAGACAAACACTTGAAGGATATCTTLGT
AGTCAAGCTCTTGCACAGGACGGAAAGACTGCTAAAGAAGAGAAAGTTGAAGGAGGC
AAAACTCACAGAAGATATAAAGTCAAGAGCAGCGACCCAGGATATGGATTCCCATAC
ACCACLGTGCTCGACGGLGTTCCTGTGGGAACAGACGAAGACGGATACGTCGTCGAA
GTTCTTATGAAAACCGGACCCCATGGAGGAGTCGACATGATGACTAGCACAGCATCA
CAAGGAAAATTCTGCGGAGTGCTTATGGATGACGGAAAAGGAAACCTAGTCGATGGA
CAAGGGAGAAAAATTACCGCCGTTATCGGCATGCTAACTCAACCGGATACCGAGTTT
AGAAGCGGACCAGGAGACGACGAGGACGACGAGTGA (A) (SEQ ID NO: 2)

or a functional equivalent thereof encoding an immunogenic polypeptide capable of inducing an immune response against Eimeria parasites.

8. A recombinant vector comprising a DNA molecule of claim 6 which recombinant vector is capable of directing the expression of the said DNA in a compatible host organism.

9. An immunogenic polypeptide according to any one of claims 1 to 3 for the immunization of a subject against coccidiosis.

10. A method for producing a polypeptide according to any one of claims 1 to 3, which method comprises:
(a) culturing a transformed microorganism containing a recombinant vector comprising a DNA having a nucleotide sequence encoding the said polypeptide under conditions in which the DNA is expressed; and
(b) isolating the polypeptide or fragment from the culture.

11. A method for producing a recombinant vector comprising a DNA having a nucleotide sequence encoding a polypeptide according to any one of claims 1 to 3, which method comprises:
(a) inserting a DNA having a nucleotide sequence encoding the said polypeptide into a vector;
(b) replicating the said vector in a microorganism; and
(c) isolating the recombinant vector from the microorganism.

12. A method for producing a recombinant virus comprising a DNA having a nucleotide sequence encoding a polypeptide according to any one of claims 1 to 3, which method comprises:
(a) inserting a DNA having a nucleotide sequence encoding the said polypeptide into the genome of a virus without inhibiting viral maturation and infectivity;
(b) amplifying the said recombinant virus in a cell culture; and
(c) purifying the recombinant virus from the culture medium.

13. A method for producing a transformed microorganism capable of producing a polypeptide according to any one of claims 1 to 3, which method comprises:
(a) transforming a microorganism with a recombinant vector comprising a DNA having a nucleotide

sequence encoding the said polypeptide; and
(b) growing the transformed microorganism in a fermentation broth.

14. A vaccine for protecting a subject against coccidiosis comprising a polypeptide according to any one of claims 1 to 3 and a physiologically acceptable carrier or adjuvant.

15. A vaccine for protecting a subject against coccidiosis containing a recombinant virus comprising a DNA having a nucleotide sequence encoding a polypeptide according to any one of claims 1 to 3, which recombinant virus is capable of directing the expression of the DNA in a compatible host organism, and a physiologically acceptable carrier or adjuvant.

16. The use of a polypeptide according to any one of claims 1 to 3 for the preparation of a vaccine capable of protecting a subject against coccidiosis.

Claims for the following Contracting States : GR, ES

1. A process for the preparation of an immunogenic polypeptide having the amino acid sequence

M A K S M L S G I V F A G L V A A A A A
S S A N S A A N V S V L E S G P A V Q E
V P A R T V T A R L A K P L L L L S A L
A A T L A A A F L V L Q C F N I I S S N
N Q Q T S V R R L A A G G A C G D E E D
A D E G T S Q Q A S R R R R K P D T P A
A D K Y D F V G G T P V S V T E P N V D
E V L I Q I R N K Q I F L K N P W T G Q
E E Q V L V L E R Q S E E P I L I V A R
T R Q T L E G Y L G S Q A L A Q D G K T
A K E E K V E G G K T H R R Y K V K S S
D P G Y G F P Y T T V L D G V P V G T D
E D G Y V V E V L M K T G P H G G V D M
M T S T A S Q G K F C G V L M D D G K G
N L V D G Q G R K I T A V I G M L T Q P
D T E F R S G P G D D E D D E (1)

(SEQID NO.1)

or fragments thereof which fragments are capable of inducing an immune response against Eimeria parasites, said polypeptide and fragments thereof being substantially free of other proteins produced by Eimeria parasites, which process comprises:
(a) culturing a transformed microorganism containing a recombinant vector comprising a DNA having a nucleotide sequence encoding the said polypeptide under conditions in which the DNA is expressed; and
(b) isolating the polypeptide or fragment from the culture.

2. A process according to claim 1 wherein the transformed microorganism contains a recombinant vector comprising a DNA sequence encoding a fragment of the immunogenic polypeptide of claim 1 which fragment has the sequence of (SEQ ID NO.1) but lacks the signal peptide sequence.

3. A process according to claim 1 wherein the immunogenic polypeptide has an apparent molecular weight of 23 kilodaltons as measured by SDS-PAGE.

4. A process according to claim 1 wherein the immunogenic polypeptide is capable of inducing a T-cell mediated immune response.

5. A process for the preparation of an immunogenic peptide comprising an amino acid sequence selected from the group of

SNNQQTSV (2) (SEQ ID NO. 3),
CGDEEDADEGTSQQASRRRRKPDTPAADK (3)(SEQ ID NO. 4).
PNV (4)(SEQ ID NO: 5),
RNKQIF (5)(SEQ ID NO: 6),
NPWTGQEE (6)(SEQ ID NO: 7),
RQSEE (7)(SEQ ID NO: 8),
TRQTLE (8)(SEQ ID NO: 9),
QDGKTAKEEKVEGGKTHRRYKVKSSDPGYG (9)(SEQ ID NO: 10),
TDEDG (10)(SEQ ID NO: 11),
TGPHG (11)(SEQ ID NO: 12),
ASQGK (12)(SEQ ID NO: 13),
DDGKGNLVDGQGRK (13)(SEQ ID NO: 14), or
TQPDTEFRSGPGDDEDDE (14)(SEQ ID NO: 15)

which process is characterized by using conventional peptide synthetic methods for synthesizing the peptide and purifying the peptide to essential homogeneity

6. A process for the preparation of a recombinant vector comprising a DNA having a nucleotide sequence encoding a polypeptide as defined in claim 1 or 2, which process comprises:
(a) inserting a DNA having a nucleotide sequence encoding the said polypeptide into a vector;
(b) replicating the said vector in a microorganism; and
(c) isolating the recombinant vector from the microorganism.

7. A process for the preparation of a recombinant virus comprising a DNA having a nucleotide sequence encoding a polypeptide as defined in claim 1 or 2, which process comprises:
(a) inserting a DNA having a nucleotide sequence encoding the said polypeptide into the genome of a virus without inhibiting viral maturation and infectivity;
(b) amplifying the said recombinant virus in a cell culture; and
(c) purifying the recombinant virus from the culture medium.

8. A process for the preparation of a transformed microorganism capable of producing a polypeptide as defined in claims 1 or 2, which process comprises:
(a) transforming a microorganism with a recombinant vector comprising a DNA having a nucleotide sequence encoding the said polypeptide; and
(b) growing the transformed microorganism in a fermentation broth.

9. A process for the preparation of a vaccine for the immunization of a subject against coccidiosis, which process comprises mixing an immunogenic polypeptide as defined in claim 1 or 2 with a pharmaceutically acceptable carrier.

10. A vaccine for the immunization of a subject against coccidiosis comprising one or more immunogenic

polypeptides as defined in claim 1 or 2 and a physiologically acceptable carrier or adjuvant.

11. A vaccine for protecting a subject against coccidiosis containing a recombinant virus comprising a DNA having a nucleotide sequence encoding an immunogenic polypeptide as defined in claim 1 or 2, which recombinant virus is capable of directing the expression of the DNA in a compatible host organism, and a physiologically acceptable carrier or adjuvant.

12. The use of an immunogenic polypeptide as defined in claim 1 or 2 for the preparation of a vaccine capable of protecting s subject against coccidiosis.

FIG 1 a

```
GCTTTTGCGTCGGAGATAGTCGTTGTGTGTTTGCGCGATCACCCGCGAACTTCTCTACCA
1--------+---------+---------+---------+---------+---------+ 60
CGAAAACGCAGCCTCTATCAGCAACACACAAACGCGCTAGTGGGCGCTTGAAGAGATGGT

ACTGAAAATGGCTAAGTCTATGCTTTCTGGAATTGTTTTTGCTGGTCTTGTTGCTGCTGC
---------+---------+---------+---------+---------+---------+ 120
TGACTTTTACCGATTCAGATACGAAAGACCTTAACAAAAACGACCAGAACAACGACGACG

      M  A  K  S  M  L  S  G  I  V  F  A  G  L  V  A  A  A

AGCGGCCAGTTCGGCCAACAGCGCCGCCAACGTCTCCGTTTTGGAGAGTGGGCCCGCTGT
---------+---------+---------+---------+---------+---------+ 180
TCGCCGGTCAAGCCGGTTGTCGCGGCGGTTGCAGAGGCAAAACCTCTCACCCGGGCGACA

 A  A  S  S  A  N  S  A  A  N  V  S  V  L  E  S  G  P  A  V

GCAGGAAGTGCCAGCGCGCACGGTCACAGCTCGCCTGGCGAAGCCTTTGCTGCTTCTTTC
---------+---------+---------+---------+---------+---------+ 240
CGTCCTTCACGGTCGCGCGTGCCAGTGTCGAGCGGACCGCTTCGGAAACGACGAAGAAAG

 Q  E  V  P  A  R  T  V  T  A  R  L  A  K  P  L  L  L  L  S

TGCTCTTGCTGCGACTTTGGCAGCAGCTTTCCTCGTTTTGCAATGCTTCAACATCATCTC
---------+---------+---------+---------+---------+---------+ 300
ACGAGAACGACGCTGAAACCGTCGTCGAAAGGAGCAAAACGTTACGAAGTTGTAGTAGAG

 A  L  A  A  T  L  A  A  A  F  L  V  L  Q  C  F  N  I  I  S

CAGCAACAACCAGCAAACCAGCGTCAGGAGACTGGCCGCCGGAGGTGCATGCGGAGATGA
---------+---------+---------+---------+---------+---------+ 360
GTCGTTGTTGGTCGTTTGGTCGCAGTCCTCTGACCGGCGGCCTCCACGTACGCCTCTACT

 S  N  N  Q  Q  T  S  V  R  R  L  A  A  G  G  A  C  G  D  E

GGAAGATGCAGATGAGGGAACTTCACAGCAGGCCAGCCGGAGGAGGAGAAAACCTGATAC
---------+---------+---------+---------+---------+---------+ 420
CCTTCTACGTCTACTCCCTTGAAGTGTCGTCCGGTCGGCCTCCTCCTCTTTTGGACTATG

 E  D  A  D  E  G  T  S  Q  Q  A  S  R  R  R  R  K  P  D  T

CCCTGCAGCAGATAAATACGATTTTGTTGGCGGAACTCCAGTTTCGGTCACTGAGCCGAA
---------+---------+---------+---------+---------+---------+ 480
GGGACGTCGTCTATTTATGCTAAAACAACCGCCTTGAGGTCAAAGCCAGTGACTCGGCTT

 P  A  A  D  K  Y  D  F  V  G  G  T  P  V  S  V  T  E  P  N

TGTTGATGAAGTCCTTATCCAAATTAGAAATAAACAAATCTTTTTGAAGAACCCATGGAC
---------+---------+---------+---------+---------+---------+ 540
ACAACTACTTCAGGAATAGGTTTAATCTTTATTTGTTTAGAAAAACTTCTTGGGTACCTG

 V  D  E  V  L  I  Q  I  R  N  K  Q  I  F  L  K  N  P  W  T
```

FIG 1 b

```
TGGACAAGAAGAACAAGTTCTAGTACTGGAACGACAAAGTGAAGAACCCATTCTGATTGT
---------+---------+---------+---------+---------+---------+ 600
ACCTGTTCTTCTTGTTCAAGATCATGACCTTGCTGTTTCACTTCTTGGGTAAGACTAACA

 G  Q  E  E  Q  V  L  V  L  E  R  Q  S  E  E  P  I  L  I  V

GGCGAGGACAAGACAAACACTTGAAGGATATCTTGGTAGTCAAGCTCTTGCACAGGACGG
---------+---------+---------+---------+---------+---------+ 660
CCGCTCCTGTTCTGTTTGTGAACTTCCTATAGAACCATCAGTTCGAGAACGTGTCCTGCC

 A  R  T  R  Q  T  L  E  G  Y  L  G  S  Q  A  L  A  Q  D  G

AAAGACTGCTAAAGAAGAGAAAGTTGAAGGAGGCAAAACTCACAGAAGATATAAAGTCAA
---------+---------+---------+---------+---------+---------+ 720
TTTCTGACGATTTCTTCTCTTTCAACTTCCTCCGTTTTGAGTGTCTTCTATATTTCAGTT

 K  T  A  K  E  E  K  V  E  G  G  K  T  H  R  R  Y  K  V  K

GAGCAGCGACCCAGGATATGGATTCCCATACACCACGGTGCTCGACGGGGTTCCTGTGGG
---------+---------+---------+---------+---------+---------+ 780
CTCGTCGCTGGGTCCTATACCTAAGGGTATGTGGTGCCACGAGCTGCCCCAAGGACACCC

 S  S  D  P  G  Y  G  F  P  Y  T  T  V  L  D  G  V  P  V  G

AACAGACGAAGACGGATACGTCGTCGAAGTTCTTATGAAAACCGGACCCCATGGAGGAGT
---------+---------+---------+---------+---------+---------+ 840
TTGTCTGCTTCTGCCTATGCAGCAGCTTCAAGAATACTTTTGGCCTGGGGTACCTCCTCA

 T  D  E  D  G  Y  V  V  E  V  L  M  K  T  G  P  H  G  G  V

CGACATGATGACTAGCACAGCATCACAAGGAAAATTCTGCGGAGTGCTTATGGATGACGG
---------+---------+---------+---------+---------+---------+ 900
GCTGTACTACTGATCGTGTCGTAGTGTTCCTTTTAAGACGCCTCACGAATACCTACTGCC

 D  M  M  T  S  T  A  S  Q  G  K  F  C  G  V  L  M  D  D  G

AAAAGGAAACCTAGTCGATGGACAAGGGAGAAAAATTACCGCCGTTATCGGCATGCTAAC
---------+---------+---------+---------+---------+---------+ 960
TTTTCCTTTGGATCAGCTACCTGTTCCCTCTTTTTAATGGCGGCAATAGCCGTACGATTG

 K  G  N  L  V  D  G  Q  G  R  K  I  T  A  V  I  G  M  L  T

TCAACCGGATACCGAGTTTAGAAGCGGACCAGGAGACGACGAGGACGACGAGTGAGTGAG
---------+---------+---------+---------+---------+---------+ 1020
AGTTGGCCTATGGCTCAAATCTTCGCCTGGTCCTCTGCTGCTCCTGCTGCTCACTCACTC

 Q  P  D  T  E  F  R  S  G  P  G  D  D  E  D  D  E  -

CGGAGTTGGCTTTTGTCCCTGTTGATGCCGTTGCCCACTTTCGCAGCTTGCTTGTTTCCT
---------+---------+---------+---------+---------+---------+ 1080
GCCTCAACCGAAAACAGGGACAACTACGGCAACGGGTGAAAGCGTCGAACGAACAAAGGA
```

FIG 1 c

```
GGGCTTGCCTGTGCCGCGACATGCGCTTGGCGTTCCGCCTGAGTTCTTTCGGACTGTTTT
---------+---------+---------+---------+---------+---------+ 1140
CCCGAACGGACACGGCGCTGTACGCGAACCGCAAGGCGGACTCAAGAAAGCCTGACAAAA

AACTTTTAATTCATTTTCTACTGCGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
---------+---------+---------+---------+---------+------- 1197
TTGAAAATTAAGTAAAAGATGACGCCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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FIG 2

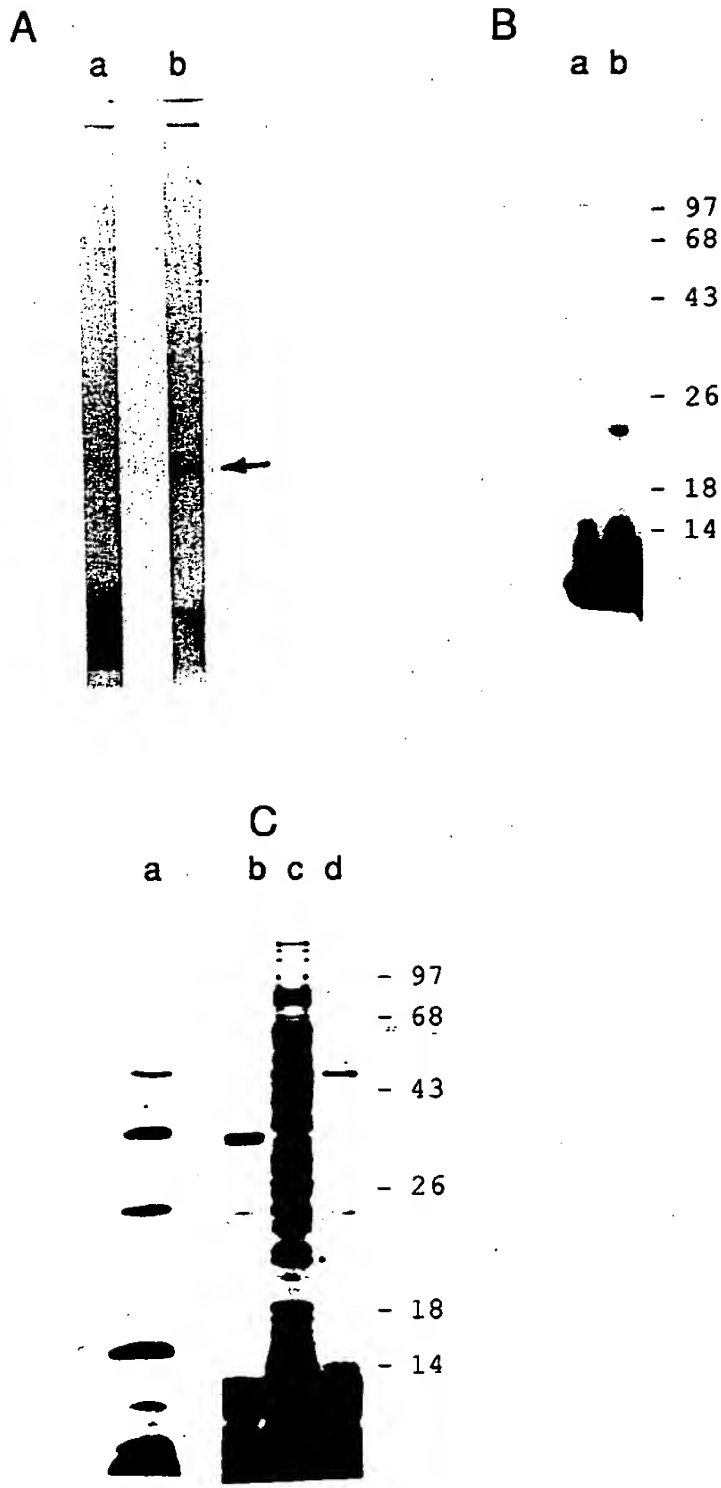


FIG 3

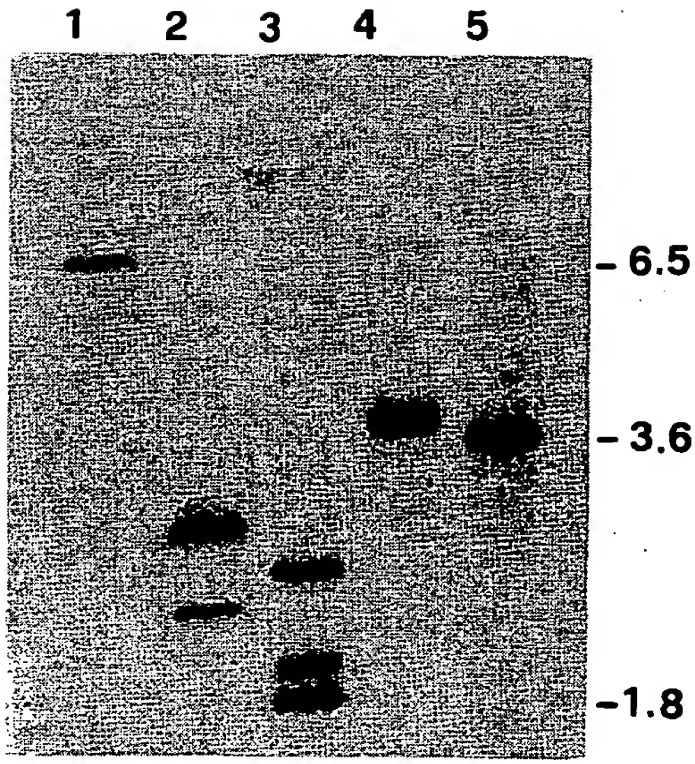
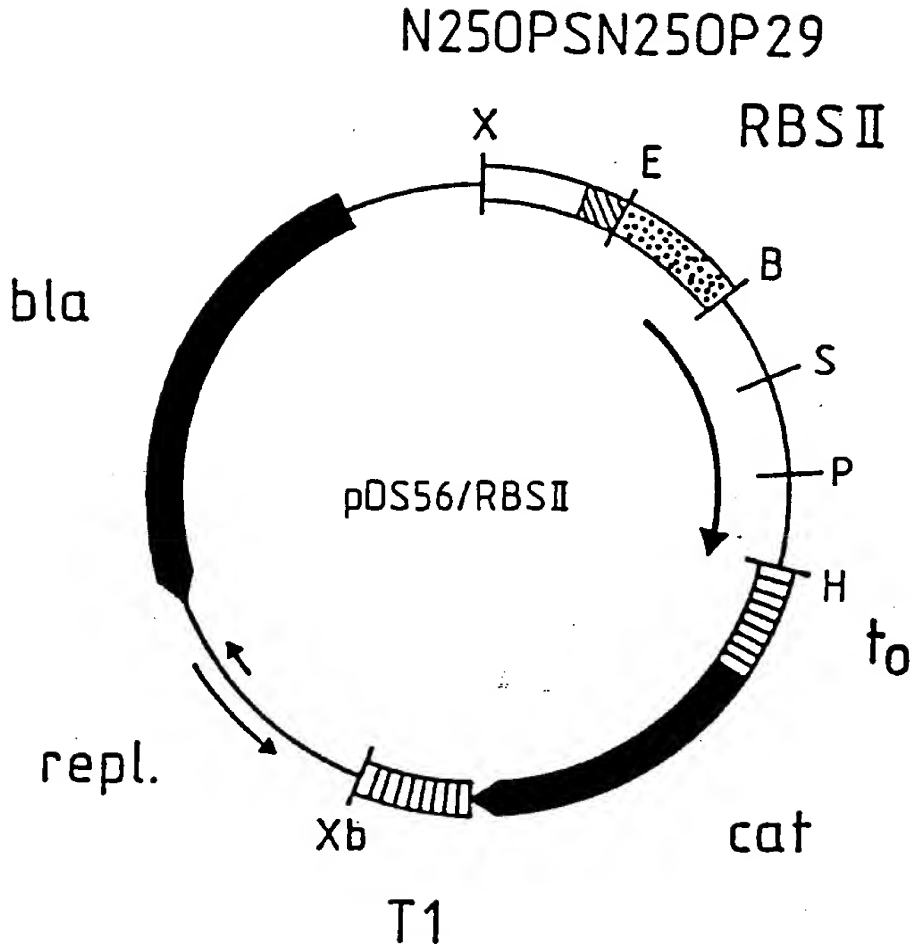


FIG 4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RBSII
E
B
S
P
H
bla
pOS56/RBSII
to
repl.
Xb
cat
T1

FIG 5 a

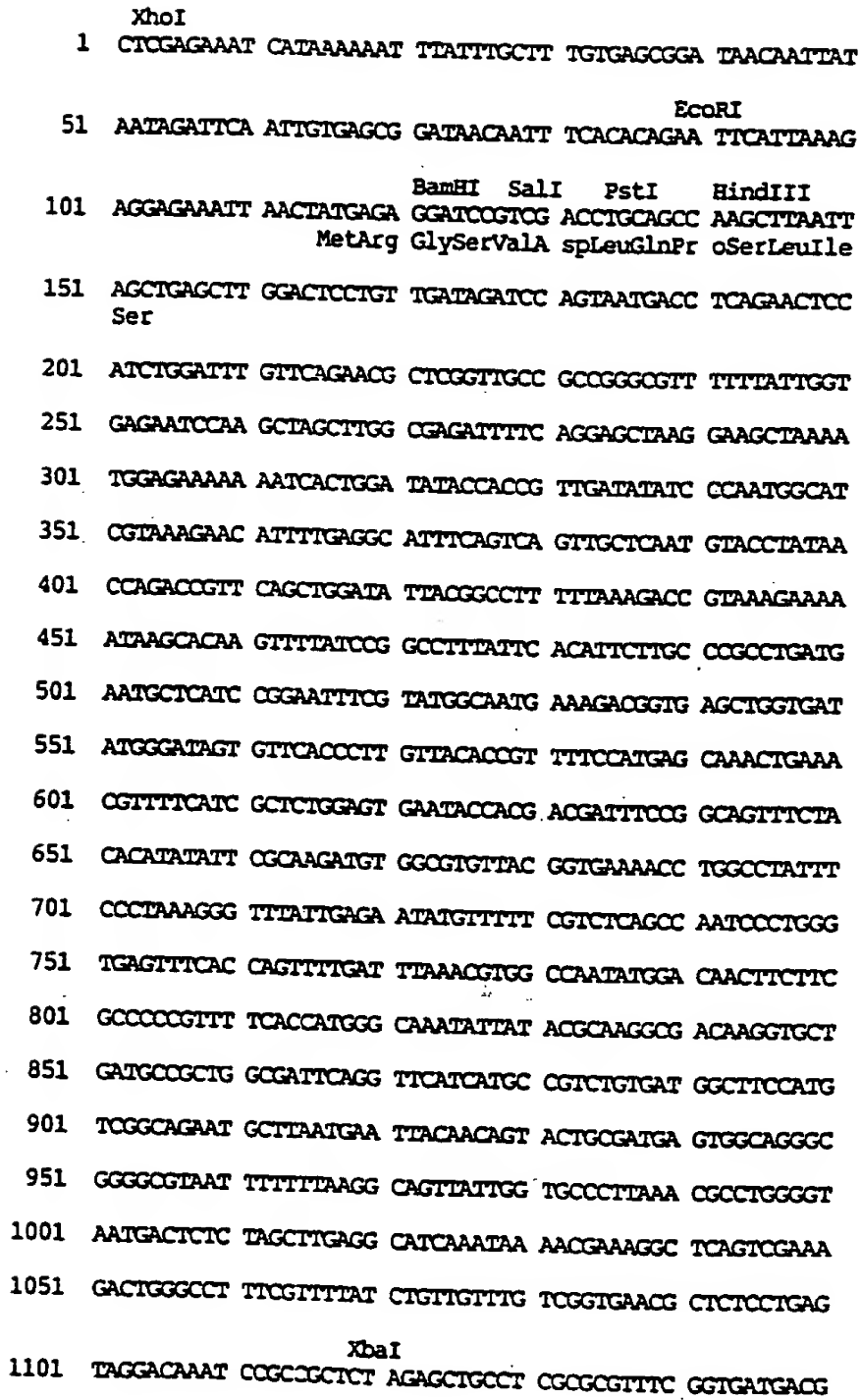

XhoI
1 CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT
EcoRI
51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
BamHI SalI PstI HindIII
101 AGGAGAAATT AACTATGAGA GGATCCGTCG ACCTGCAGCC AAGCTTAATT
MetArg GlySerValA spLeuGlnPr oSerLeuIle
151 AGCTGAGCTT GGACTCCTGT TGATAGATCC AGTAATGACC TCAGAACTCC
Ser
201 ATCTGGATTT GTTCAGAACG CTCGGTTGCC GCCGGGCGTT TTTTATTGGT
251 GAGAATCCAA GCTAGCTTGG CGAGATTTTC AGGAGCTAAG GAAGCTAAAA
301 TGGAGAAAAA AATCACTGGA TATACCACCG TTGATATATC CCAATGGCAT
351 CGTAAAGAAC ATTTTGAGGC ATTTCAGTCA GTTGCTCAAT GTACCTATAA
401 CCAGACCGTT CAGCTGGATA TTACGGCCTT TTTAAAGACC GTAAAGAAAA
451 ATAAGCACAA GTTTTATCCG GCCTTTATTC ACATTCTTGC CCGCCTGATG
501 AATGCTCATC CGGAATTTCG TATGGCAATG AAAGACGGTG AGCTGGTGAT
551 ATGGGATAGT GTTCACCCTT GTTACACCGT TTTCCATGAG CAAACTGAAA
601 CGTTTTCATC GCTCTGGAGT GAATACCACG ACGATTTCCG GCAGTTTCTA
651 CACATATATT CGCAAGATGT GGCGTGTTAC GGTGAAAACC TGGCCTATTT
701 CCCTAAAGGG TTTATTGAGA ATATGTTTTT CGTCTCAGCC AATCCCTGGG
751 TGAGTTTCAC CAGTTTTGAT TTAAACGTGG CCAATATGGA CAACTTCTTC
801 GCCCCCGTTT TCACCATGGG CAAATATTAT ACGCAAGGCG ACAAGGTGCT
851 GATGCCGCTG GCGATTCAGG TTCATCATGC CGTCTGTGAT GGCTTCCATG
901 TCGGCAGAAT GCTTAATGAA TTACAACAGT ACTGCGATGA GTGGCAGGGC
951 GGGGCGTAAT TTTTTTAAGG CAGTTATTGG TGCCCTTAAA CGCCTGGGGT
1001 AATGACTCTC TAGCTTGAGG CATCAAATAA AACGAAAGGC TCAGTCGAAA
1051 GACTGGGCCT TTCGTTTTAT CTGTTGTTTG TCGGTGAACG CTCTCCTGAG
XbaI
1101 TAGGACAAAT CCGCCGCTCT AGAGCTGCCT CGCGCGTTTC GGTGATGACG

FIG 5 b

1151 GTGAAAACCT CTGACACATG CAGCTCCCGG AGACGGTCAC AGCTTGTCTG

1201 TAAGCGGATG CCGGGAGCAG ACAAGCCCGT CAGGGCGCGT CAGCGGGTGT

1251 TGGCGGGTGT CGGGGCGCAG CCATGACCCA GTCACGTAGC GATAGCGGAG

1301 TGTATACTGG CTTAACTATG CGGCATCAGA GCAGATTGTA CTGAGAGTGC

1351 ACCATATGCG GTGTGAAATA CCGCACAGAT GCGTAAGGAG AAAATACCGC

1401 ATCAGGCGCT CTTCCGCTTC CTCGCTCACT GACTCGCTGC GCTCGGTCTG

1451 TCGGCTGCGG CGAGCGGTAT CAGCTCACTC AAAGGCGGTA ATACGGTTAT

1501 CCACAGAATC AGGGGATAAC GCAGGAAAGA ACATGTGAGC AAAAGGCCAG

1551 CAAAAGGCCA GGAACCGTAA AAAGGCCGCG TTGCTGGCGT TTTTCCATAG

1601 GCTCCGCCCC CCTGACGAGC ATCACAAAAA TCGACGCTCA AGTCAGAGGT

1651 GGCGAAACCC GACAGGACTA TAAAGATACC AGGCGTTTCC CCCTGGAAGC

1701 TCCCTCGTGC GCTCTCCTGT TCCGACCCTG CCGCTTACCG GATACCTGTC

1751 CGCCTTTCTC CCTTCGGGAA GCGTGGCGCT TTCTCAATGC TCACGCTGTA

1801 GGTATCTCAG TTCGGTGTAG GTCGTTCGCT CCAAGCTGGG CTGTGTGCAC

1851 GAACCCCCCG TTCAGCCCGA CCGCTGCGCC TTATCCGGTA ACTATCGTCT

1901 TGAGTCCAAC CCGGTAAGAC ACGACTTATC GCCACTGGCA GCAGCCACTG

1951 GTAACAGGAT TAGCAGAGCG AGGTATGTAG GCGGTGCTAC AGAGTTCTTG

2001 AAGTGGTGGC CTAACTACGG CTACACTAGA AGGACAGTAT TTGGTATCTG

2051 CGCTCTGCTG AAGCCAGTTA CCTTCGGAAA AAGAGTTGGT AGCTCTTGAT

2101 CCGGCAAACA AACCACCGCT GGTAGCGGTG GTTTTTTTGT TTGCAAGCAG

2151 CAGATTACGC GCAGAAAAAA AGGATCTCAA GAAGATCCTT TGATCTTTTC

2201 TACGGGGTCT GACGCTCAGT GGAACGAAAA CTCACGTTAA GGGATTTTGG

2251 TCATGAGATT ATCAAAAAGG ATCTTCACCT AGATCCTTTT AAATTAAAAA

2301 TGAAGTTTTA AATCAATCTA AAGTATATAT GAGTAAACTT GGTCTGACAG

2351 TTACCAATGC TTAATCAGTG AGGCACCTAT CTCAGCGATC TGTCTATTTC

2401 GTTCATCCAT AGCTGCCTGA CTCCCCGTCG TGTAGATAAC TACGATACGG

2451 GAGGGCTTAC CATCTGGCCC CAGTGCTGCA ATGATACCGC GAGACCCACG

FIG 5 c

2501 CTCACCGGCT CCAGATTTAT CAGCAATAAA CCAGCCAGCC GGAAGGGCCG

2551 AGCGCAGAAG TGGTCCTGCA ACTTTATCCG CCTCCATCCA GTCTATTAAT

2601 TGTTGCCGGG AAGCTAGAGT AAGTAGTTCG CCAGTTAATA GTTTGCGCAA

2651 CGTTGTTGCC ATTGCTACAG GCATCGTGGT GTCACGCTCG TCGTTTGGTA

2701 TGGCTTCATT CAGCTCCGGT TCCCAACGAT CAAGGCGAGT TACATGATCC

2751 CCCATGTTGT GCAAAAAAGC GGTTAGCTCC TTCGGTCCTC CGATCGTTGT

2801 CAGAAGTAAG TTGGCCGCAG TGTTATCACT CATGGTTATG GCAGCACTGC

2851 ATAATTCTCT TACTGTCATG CCATCCGTAA GATGCTTTTC TGTGACTGGT

2901 GAGTACTCAA CCAAGTCATT CTGAGAATAG TGTATGCGGC GACCGAGTTG

2951 CTCTTGCCCG GCGTCAATAC GGGATAATAC CGCGCCACAT AGCAGAACTT

3001 TAAAAGTGCT CATCATTGGA AAACGTTCTT CGGGGCGAAA ACTCTCAAGG

3051 ATCTTACCGC TGTTGAGATC CAGTTCGATG TAACCCACTC GTGCACCCAA

3101 CTGATCTTCA GCATCTTTTA CTTTCACCAG CGTTTCTGGG TGAGCAAAAA

3151 CAGGAAGGCA AAATGCCGCA AAAAAGGGAA TAAGGGCGAC ACGGAAATGT

3201 TGAATACTCA TACTCTTCCT TTTTCAATAT TATTGAAGCA TTTATCAGGG

3251 TTATTGTCTC ATGAGCGGAT ACATATTTGA ATGTATTTAG AAAAATAAAC

3301 AAATAGGGGT TCCGCGCACA TTTCCCCGAA AAGTGCCACC TGACGTCTAA

3351 GAAACCATTA TTATCATGAC ATTAACCTAT AAAAATAGGC GTATCACGAG

3401 GCCCTTTCGT CTTCAC

FIG 6

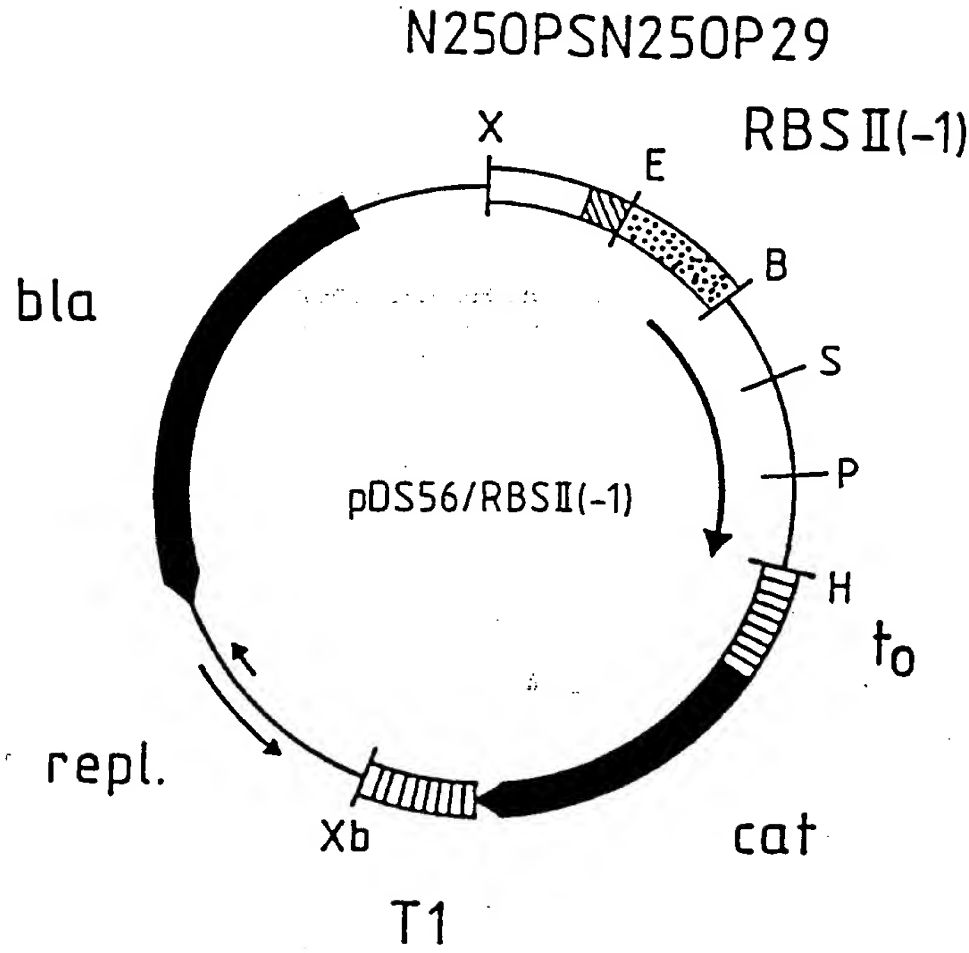

N25OPSN25OP29
X
E
RBSII(-1)
B
bla
S
P
pDS56/RBSII(-1)
H
to
repl.
Xb
cat
T1

FIG 7 a

```
      XhoI
1     CTCGAGAAAT CATAAAAAAT TTATTTGCTT TGTGAGCGGA TAACAATTAT

                                                 EcoRI
51    AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG

                          BamHI  SalI   PstI   HindIII
101   AGGAGAAATT AACTATGAGG GATCCGTCGA CCTGCAGCCA AGCTTAATTA
                    MetArg AspProSerT hrCysSerGl nAla

151   GCTGAGCTTG GACTCCTGTT GATAGATCCA GTAATGACCT CAGAACTCCA

201   TCTGGATTTG TTCAGAACGC TCGGTTGCCG CCGGGCGTTT TTTATTGGTG

251   AGAATCCAAG CTAGCTTGGC GAGATTTTCA GGAGCTAAGG AAGCTAAAAT

301   GGAGAAAAAA ATCACTGGAT ATACCACCGT TGATATATCC CAATGGCATC

351   GTAAAGAACA TTTTGAGGCA TTTCAGTCAG TTGCTCAATG TACCTATAAC

401   CAGACCGTTC AGCTGGATAT TACGGCCTTT TTAAAGACCG TAAAGAAAAA

451   TAAGCACAAG TTTTATCCGG CCTTTATTCA CATTCTTGCC CGCCTGATGA

501   ATGCTCATCC GGAATTTCGT ATGGCAATGA AAGACGGTGA GCTGGTGATA

551   TGGGATAGTG TTCACCCTTG TTACACCGTT TTCCATGAGC AAACTGAAAC

601   GTTTTCATCG CTCTGGAGTG AATACCACGA CGATTTCCGG CAGTTTCTAC

651   ACATATATTC GCAAGATGTG GCGTGTTACG GTGAAAACCT GGCCTATTTC

701   CCTAAAGGGT TTATTGAGAA TATGTTTTTC GTCTCAGCCA ATCCCTGGGT

751   GAGTTTCACC AGTTTTGATT TAAACGTGGC CAATATGGAC AACTTCTTCG

801   CCCCCGTTTT CACCATGGGC AAATATTATA CGCAAGGCGA CAAGGTGCTG

851   ATGCCGCTGG CGATTCAGGT TCATCATGCC GTCTGTGATG GCTTCCATGT

901   CGGCAGAATG CTTAATGAAT TACAACAGTA CTGCGATGAG TGGCAGGGCG

951   GGGCGTAATT TTTTTAAGGC AGTTATTGGT GCCCTTAAAC GCCTGGGGTA

1001  ATGACTCTCT AGCTTGAGGC ATCAAATAAA ACGAAAGGCT CAGTCGAAAG

1051  ACTGGGCCTT TCGTTTTATC TGTTGTTTGT CGGTGAACGC TCTCCTGAGT

                    XbaI
1101  AGGACAAATC CGCCGCTCTA GAGCTGCCTC GCGCGTTTCG GTGATGACGG
```

FIG 7 b

1151 TGAAAACCTC TGACACATGC AGCTCCCGGA GACGGTCACA GCTTGTCTGT

1201 AAGCGGATGC CGGGAGCAGA CAAGCCCGTC AGGGCGCGTC AGCGGGTGTT

1251 GGCGGGTGTC GGGGCGCAGC CATGACCCAG TCACGTAGCG ATAGCGGAGT

1301 GTATACTGGC TTAACTATGC GGCATCAGAG CAGATTGTAC TGAGAGTGCA

1351 CCATATGCGG TGTGAAATAC CGCACAGATG CGTAAGGAGA AAATACCGCA

1401 TCAGGCGCTC TTCCGCTTCC TCGCTCACTG ACTCGCTGCG CTCGGTCTGT

1451 CGGCTGCGGC GAGCGGTATC AGCTCACTCA AAGGCGGTAA TACGGTTATC

1501 CACAGAATCA GGGGATAACG CAGGAAAGAA CATGTGAGCA AAAGGCCAGC

1551 AAAAGGCCAG GAACCGTAAA AAGGCCGCGT TGCTGGCGTT TTTCCATAGG

1601 CTCCGCCCCC CTGACGAGCA TCACAAAAAT CGACGCTCAA GTCAGAGGTG

1651 GCGAAACCCG ACAGGACTAT AAAGATACCA GGCGTTTCCC CCTGGAAGCT

1701 CCCTCGTGCG CTCTCCTGTT CCGACCCTGC CGCTTACCGG ATACCTGTCC

1751 GCCTTTCTCC CTTCGGGAAG CGTGGCGCTT TCTCAATGCT CACGCTGTAG

1801 GTATCTCAGT TCGGTGTAGG TCGTTCGCTC CAAGCTGGGC TGTGTGCACG

1851 AACCCCCCGT TCAGCCCGAC CGCTGCGCCT TATCCGGTAA CTATCGTCTT

1901 GAGTCCAACC CGGTAAGACA CGACTTATCG CCACTGGCAG CAGCCACTGG

1951 TAACAGGATT AGCAGAGCGA GGTATGTAGG CGGTGCTACA GAGTTCTTGA

2001 AGTGGTGGCC TAACTACGGC TACACTAGAA GGACAGTATT TGGTATCTGC

2051 GCTCTGCTGA AGCCAGTTAC CTTCGGAAAA AGAGTTGGTA GCTCTTGATC

2101 CGGCAAACAA ACCACCGCTG GTAGCGGTGG TTTTTTTGTT TGCAAGCAGC

2151 AGATTACGCG CAGAAAAAAA GGATCTCAAG AAGATCCTTT GATCTTTTCT

2201 ACGGGGTCTG ACGCTCAGTG GAACGAAAAC TCACGTTAAG GGATTTTGGT

2251 CATGAGATTA TCAAAAAGGA TCTTCACCTA GATCCTTTTA AATTAAAAAT

2301 GAAGTTTTAA ATCAATCTAA AGTATATATG AGTAAACTTG GTCTGACAGT

2351 TACCAATGCT TAATCAGTGA GGCACCTATC TCAGCGATCT GTCTATTTCG

2401 TTCATCCATA GCTGCCTGAC TCCCCGTCGT GTAGATAACT ACGATACGGG

2451 AGGGCTTACC ATCTGGCCCC AGTGCTGCAA TGATACCGCG AGACCCACGC

FIG 7 c

2501 TCACCGGCTC CAGATTTATC AGCAATAAAC CAGCCAGCCG GAAGGGCCGA

2551 GCGCAGAAGT GGTCCTGCAA CTTTATCCGC CTCCATCCAG TCTATTAATT

2601 GTTGCCGGGA AGCTAGAGTA AGTAGTTCGC CAGTTAATAG TTTGCGCAAC

2651 GTTGTTGCCA TTGCTACAGG CATCGTGGTG TCACGCTCGT CGTTTGGTAT

2701 GGCTTCATTC AGCTCCGGTT CCCAACGATC AAGGCGAGTT ACATGATCCC

2751 CCATGTTGTG CAAAAAAGCG GTTAGCTCCT TCGGTCCTCC GATCGTTGTC

2801 AGAAGTAAGT TGGCCGCAGT GTTATCACTC ATGGTTATGG CAGCACTGCA

2851 TAATTCTCTT ACTGTCATGC CATCCGTAAG ATGCTTTTCT GTGACTGGTG

2901 AGTACTCAAC CAAGTCATTC TGAGAATAGT GTATGCGGCG ACCGAGTTGC

2951 TCTTGCCCGG CGTCAATACG GGATAATACC GCGCCACATA GCAGAACTTT

3001 AAAAGTGCTC ATCATTGGAA AACGTTCTTC GGGGCGAAAA CTCTCAAGGA

3051 TCTTACCGCT GTTGAGATCC AGTTCGATGT AACCCACTCG TGCACCCAAC

3101 TGATCTTCAG CATCTTTTAC TTTCACCAGC GTTTCTGGGT GAGCAAAAAC

3151 AGGAAGGCAA AATGCCGCAA AAAAGGGAAT AAGGGCGACA CGGAAATGTT

3201 GAATACTCAT ACTCTTCCTT TTTCAATATT ATTGAAGCAT TTATCAGGGT

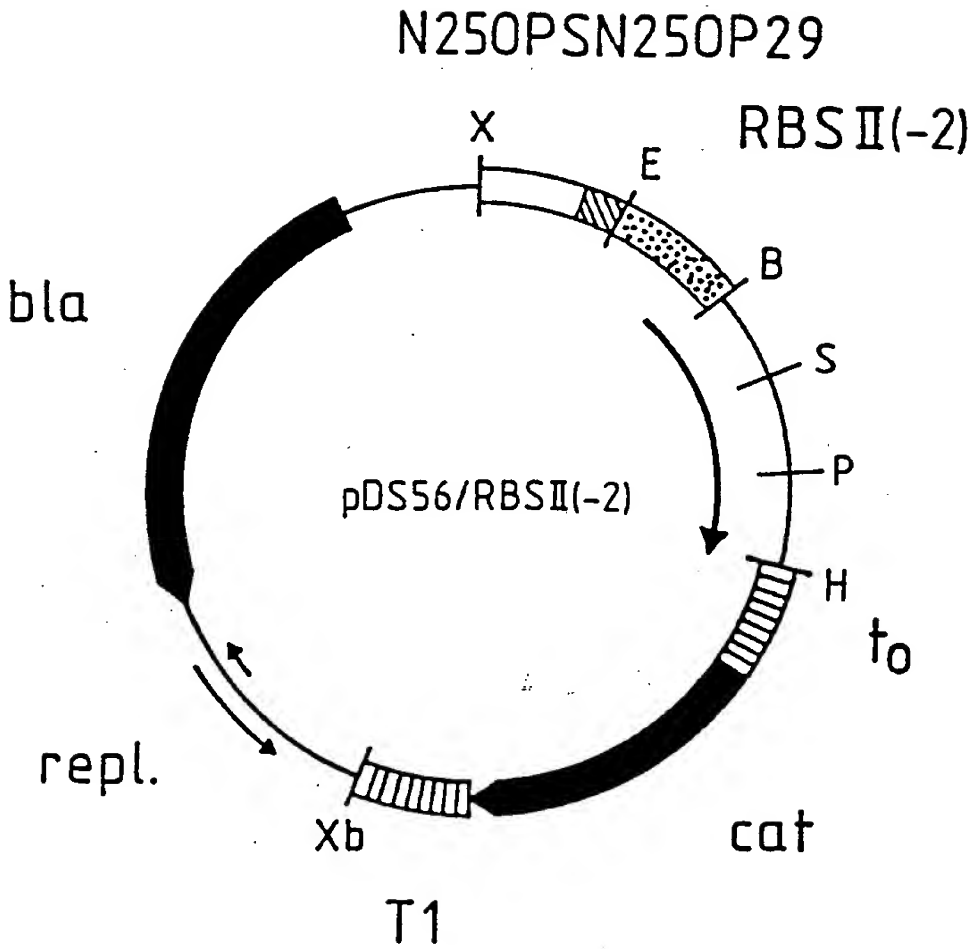
3251 TATTGTCTCA TGAGCGGATA CATATTTGAA TGTATTTAGA AAAATAAACA

3301 AATAGGGGTT CCGCGCACAT TTCCCCGAAA AGTGCCACCT GACGTCTAAG

3351 AAACCATTAT TATCATGACA TTAACCTATA AAAATAGGCG TATCACGAGG

3401 CCCTTTCGTC TTCAC

FIG 8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X
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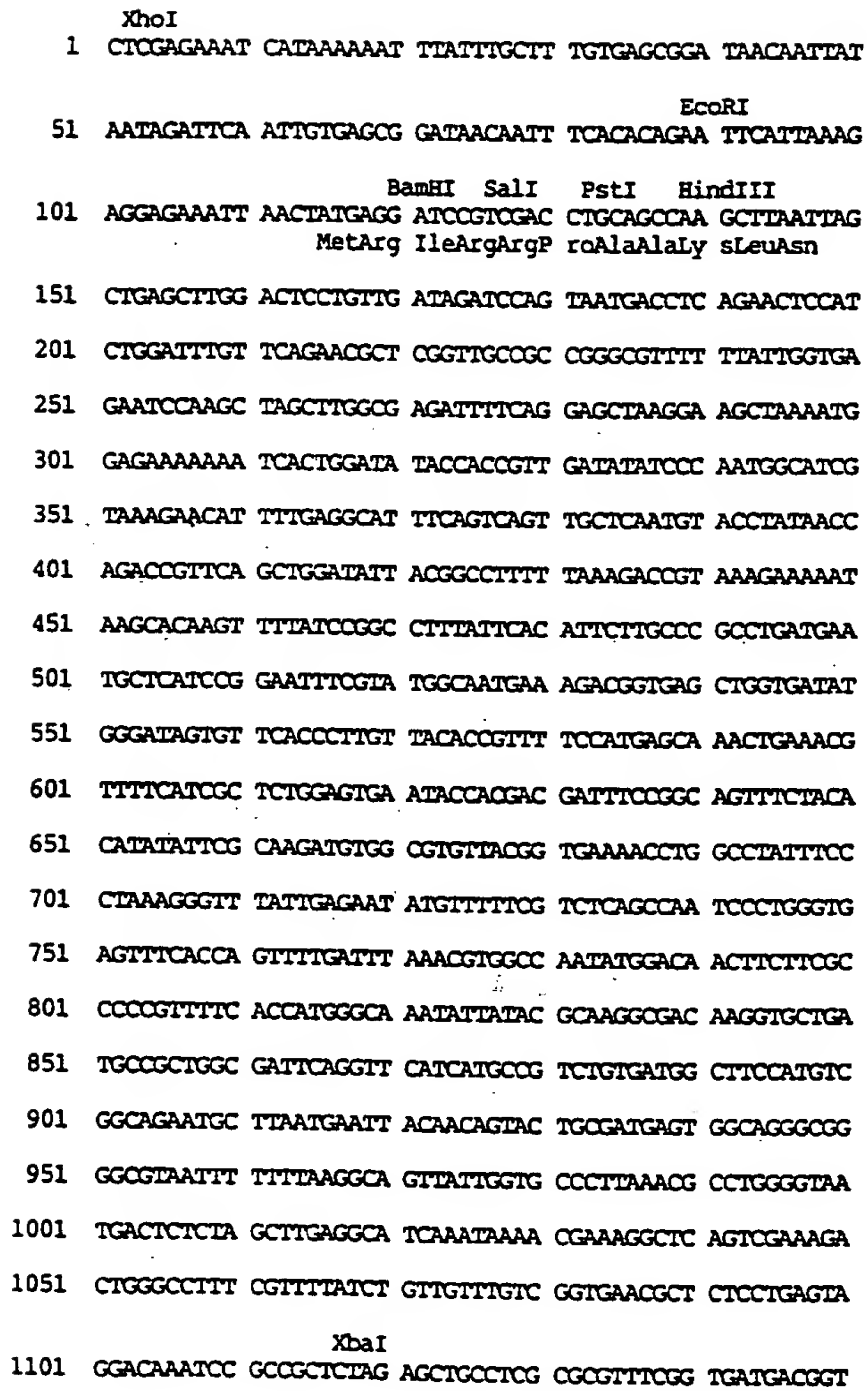
E
B
S
P
H
bla
pDS56/RBSII(-2)
repl.
Xb
cat
T1

FIG 9 a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EcoRI
51 AATAGATTCA ATTGTGAGCG GATAACAATT TCACACAGAA TTCATTAAAG
BamHI SalI PstI HindIII
101 AGGAGAAATT AACTATGAGG ATCCGTCGAC CTGCAGCCAA GCTTAATTAG
MetArg IleArgArgP roAlaAlaLy sLeuAsn
151 CTGAGCTTGG ACTCCTGTTG ATAGATCCAG TAATGACCTC AGAACTCCAT
201 CTGGATTTGT TCAGAACGCT CGGTTGCCGC CGGGCGTTTT TTATTGGTGA
251 GAATCCAAGC TAGCTTGGCG AGATTTTCAG GAGCTAAGGA AGCTAAAATG
301 GAGAAAAAAA TCACTGGATA TACCACCGTT GATATATCCC AATGGCATCG
351 TAAAGAACAT TTTGAGGCAT TTCAGTCAGT TGCTCAATGT ACCTATAACC
401 AGACCGTTCA GCTGGATATT ACGGCCTTTT TAAAGACCGT AAAGAAAAAT
451 AAGCACAAGT TTTATCCGGC CTTTATTCAC ATTCTTGCCC GCCTGATGAA
501 TGCTCATCCG GAATTTCGTA TGGCAATGAA AGACGGTGAG CTGGTGATAT
551 GGGATAGTGT TCACCCTTGT TACACCGTTT TCCATGAGCA AACTGAAACG
601 TTTTCATCGC TCTGGAGTGA ATACCACGAC GATTTCCGGC AGTTTCTACA
651 CATATATTCG CAAGATGTGG CGTGTTACGG TGAAAACCTG GCCTATTTCC
701 CTAAAGGGTT TATTGAGAAT ATGTTTTTCG TCTCAGCCAA TCCCTGGGTG
751 AGTTTCACCA GTTTTGATTT AAACGTGGCC AATATGGACA ACTTCTTCGC
801 CCCCGTTTTC ACCATGGGCA AATATTATAC GCAAGGCGAC AAGGTGCTGA
851 TGCCGCTGGC GATTCAGGTT CATCATGCCG TCTGTGATGG CTTCCATGTC
901 GGCAGAATGC TTAATGAATT ACAACAGTAC TGCGATGAGT GGCAGGGCGG
951 GGCGTAATTT TTTTAAGGCA GTTATTGGTG CCCTTAAACG CCTGGGGTAA
1001 TGACTCTCTA GCTTGAGGCA TCAAATAAAA CGAAAGGCTC AGTCGAAAGA
1051 CTGGGCCTTT CGTTTTATCT GTTGTTTGTC GGTGAACGCT CTCCTGAGTA
XbaI
1101 GGACAAATCC GCCGCTCTAG AGCTGCCTCG CGCGTTTCGG TGATGACGGT

FIG 9 b

1151 GAAAACCTCT GACACATGCA GCTCCCGGAG ACGGTCACAG CTTGTCTGTA

1201 AGCGGATGCC GGGAGCAGAC AAGCCCGTCA GGGCGCGTCA GCGGGTGTTG

1251 GCGGGTGTCG GGGCGCAGCC ATGACCCAGT CACGTAGCGA TAGCGGAGTG

1301 TATACTGGCT TAACTATGCG GCATCAGAGC AGATTGTACT GAGAGTGCAC

1351 CATATGCGGT GTGAAATACC GCACAGATGC GTAAGGAGAA AATACCGCAT

1401 CAGGCGCTCT TCCGCTTCCT CGCTCACTGA CTCGCTGCGC TCGGTCTGTC

1451 GGCTGCGGCG AGCGGTATCA GCTCACTCAA AGGCGGTAAT ACGGTTATCC

1501 ACAGAATCAG GGGATAACGC AGGAAAGAAC ATGTGAGCAA AAGGCCAGCA

1551 AAAGGCCAGG AACCGTAAAA AGGCCGCGTT GCTGGCGTTT TTCCATAGGC

1601 TCCGCCCCCC TGACGAGCAT CACAAAAATC GACGCTCAAG TCAGAGGTGG

1651 CGAAACCCGA CAGGACTATA AAGATACCAG GCGTTTCCCC CTGGAAGCTC

1701 CCTCGTGCGC TCTCCTGTTC CGACCCTGCC GCTTACCGGA TACCTGTCCG

1751 CCTTTCTCCC TTCGGGAAGC GTGGCGCTTT CTCAATGCTC ACGCTGTAGG

1801 TATCTCAGTT CGGTGTAGGT CGTTCGCTCC AAGCTGGGCT GTGTGCACGA

1851 ACCCCCCGTT CAGCCCGACC GCTGCGCCTT ATCCGGTAAC TATCGTCTTG

1901 AGTCCAACCC GGTAAGACAC GACTTATCGC CACTGGCAGC AGCCACTGGT

1951 AACAGGATTA GCAGAGCGAG GTATGTAGGC GGTGCTACAG AGTTCTTGAA

2001 GTGGTGGCCT AACTACGGCT ACACTAGAAG GACAGTATTT GGTATCTGCG

2051 CTCTGCTGAA GCCAGTTACC TTCGGAAAAA GAGTTGGTAG CTCTTGATCC

2101 GGCAAACAAA CCACCGCTGG TAGCGGTGGT TTTTTTGTTT GCAAGCAGCA

2151 GATTACGCGC AGAAAAAAAG GATCTCAAGA AGATCCTTTG ATCTTTTCTA

2201 CGGGGTCTGA CGCTCAGTGG AACGAAAACT CACGTTAAGG GATTTTGGTC

2251 ATGAGATTAT CAAAAAGGAT CTTCACCTAG ATCCTTTTAA ATTAAAAATG

2301 AAGTTTTAAA TCAATCTAAA GTATATATGA GTAAACTTGG TCTGACAGTT

2351 ACCAATGCTT AATCAGTGAG GCACCTATCT CAGCGATCTG TCTATTTCGT

2401 TCATCCATAG CTGCCTGACT CCCCGTCGTG TAGATAACTA CGATACGGGA

2451 GGGCTTACCA TCTGGCCCCA GTGCTGCAAT GATACCGCGA GACCCACGCT

FIG 9 c

2501 CACCGGCTCC AGATTTATCA GCAATAAACC AGCCAGCCGG AAGGGCCGAG

2551 CGCAGAAGTG GTCCTGCAAC TTTATCCGCC TCCATCCAGT CTATTAATTG

2601 TTGCCGGGAA GCTAGAGTAA GTAGTTCGCC AGTTAATAGT TTGCGCAACG

2651 TTGTTGCCAT TGCTACAGGC ATCGTGGTGT CACGCTCGTC GTTTGGTATG

2701 GCTTCATTCA GCTCCGGTTC CCAACGATCA AGGCGAGTTA CATGATCCCC

2751 CATGTTGTGC AAAAAAGCGG TTAGCTCCTT CGGTCCTCCG ATCGTTGTCA

2801 GAAGTAAGTT GGCCGCAGTG TTATCACTCA TGGTTATGGC AGCACTGCAT

2851 AATTCTCTTA CTGTCATGCC ATCCGTAAGA TGCTTTTCTG TGACTGGTGA

2901 GTACTCAACC AAGTCATTCT GAGAATAGTG TATGCGGCGA CCGAGTTGCT

2951 CTTGCCCGGC GTCAATACGG GATAATACCG CGCCACATAG CAGAACTTTA

3001 AAAGTGCTCA TCATTGGAAA ACGTTCTTCG GGGCGAAAAC TCTCAAGGAT

3051 CTTACCGCTG TTGAGATCCA GTTCGATGTA ACCCACTCGT GCACCCAACT

3101 GATCTTCAGC ATCTTTTACT TTCACCAGCG TTTCTGGGTG AGCAAAAACA

3151 GGAAGGCAAA ATGCCGCAAA AAAGGGAATA AGGGCGACAC GGAAATGTTG

3201 AATACTCATA CTCTTCCTTT TTCAATATTA TTGAAGCATT TATCAGGGTT

3251 ATTGTCTCAT GAGCGGATAC ATATTTGAAT GTATTTAGAA AAATAAACAA

3301 ATAGGGGTTC CGCGCACATT TCCCCGAAAA GTGCCACCTG ACGTCTAAGA

3351 AACCATTATT ATCATGACAT TAACCTATAA AAATAGGCGT ATCACGAGGC

3401 CCTTTCGTCT TCAC

FIG 10

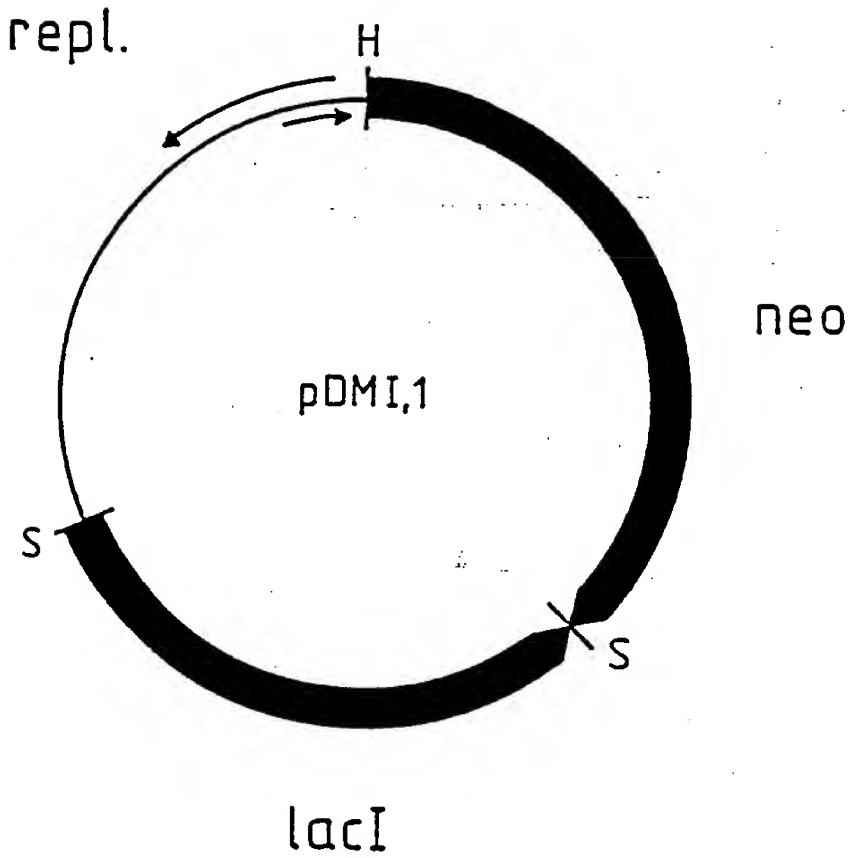


FIG 11 a

HindIII

```
   1  AAGCTTCACG CTGCCGCAAG CACTCAGGGC GCAAGGGCTG CTAAAGGAAG
  51  CGGAACACGT AGAAAGCCAG TCCGCAGAAA CGGTGCTGAC CCCGGATGAA
 101  TGTCAGCTAC TGGGCTATCT GGACAAGGGA AAACGCAAGC GCAAAGAGAA
 151  AGCAGGTAGC TTGCAGTGGG CTTACATGGC GATAGCTAGA CTGGGCGGTT
 201  TTATGGACAG CAAGCGAACC GGAATTGCCA GCTGGGGCGC CCTCTGGTAA
 251  GGTTGGGAAG CCCTGCAAAG TAAACTGGAT GGCTTTCTTG CCGCCAAGGA
 301  TCTGATGGCG CAGGGGATCA AGATCTGATC AAGAGACAGG ATGAGGATCG
 351  TTTCGCATGA TTGAACAAGA TGGATTGCAC GCAGGTTCTC CGGCCGCTTG
            Met
 401  GGTGGAGAGG CTATTCGGCT ATGACTGGGC ACAACAGACA ATCGGCTGCT
 451  CTGATGCCGC CGTGTTCCGG CTGTCAGCGC AGGGGCGCCC GGTTCTTTTT
 501  GTCAAGACCG ACCTGTCCGG TGCCCTGAAT GAACTGCAGG ACGAGGCAGC
 551  GCGGCTATCG TGGCTGGCCA CGACGGGCGT TCCTTGCGCA GCTGTGCTCG
 601  ACGTTGTCAC TGAAGCGGGA AGGGACTGGC TGCTATTGGG CGAAGTGCCG
 651  GGGCAGGATC TCCTGTCATC TCACCTTGCT CCTGCCGAGA AAGTATCCAT
 701  CATGGCTGAT GCAATGCGGC GGCTGCATAC GCTTGATCCG GCTACCTGCC
 751  CATTCGACCA CCAAGCGAAA CATCGCATCG AGCGAGCACG TACTCGGATG
 801  GAAGCCGGTC TTGTCGATCA GGATGATCTG GACGAAGAGC ATCAGGGGCT
 851  CGCGCCAGCC GAACTGTTCG CCAGGCTCAA GGCGCGCATG CCCGACGGCG
 901  AGGATCTCGT CGTGACCCAT GGCGATGCCT GCTTGCCGAA TATCATGGTG
 951  GAAAATGGCC GCTTTTCTGG ATTCATCGAC TGTGGCCGGC TGGGTGTGGC
1001  GGACCGCTAT CAGGACATAG CGTTGGCTAC CCGTGATATT GCTGAAGAGC
1051  TTGGCGGCGA ATGGGCTGAC CGCTTCCTCG TGCTTTACGG TATCGCCGCT
1101  CCCGATTCGC AGCGCATCGC CTTCTATCGC CTTCTTGACG AGTTCTTCTG
                                                    Phe
1151  AGCGGGACTC TGGGGTTCGA AATGACCGAC CAAGCGACGC CCAACCTGCC
```

FIG 11 b

```
1201  ATCACGAGAT TTCGATTCCA CCGCCGCCTT CTATGAAAGG TTGGGCTTCG
1251  GAATCGTTTT CCGGGACGCC GGCTGGATGA TCCTCCAGCG CGGGGATCTC
1301  ATGCTGGAGT TCTTCGCCCA CCCCGGGCTC GATCCCCTCG CGAGTTGGTT
1351  CAGCTGCTGC CTGAGGCTGG ACGACCTCGC GGAGTTCTAC CGGCAGTGCA
1401  AATCCGTCGG CATCCAGGAA ACCAGCAGCG GCTATCCGCG CATCCATGCC
                                                    SalI
1451  CCCGAACTGC AGGAGTGGGG AGGCACGATG GCCGCTTTGG TCGACAATTC
1501  GCGCTAACTT ACATTAATTG CGTTGCGCTC ACTGCCCGCT TTCCAGTCGG
                                      (Gln)
1551  GAAACCTGTC GTGCCAGCTG CATTAATGAA TCGGCCAACG CGCGGGGAGA
1601  GGCGGTTTGC GTATTGGGCG CCAGGGTGGT TTTTCTTTTC ACCAGTGAGA
1651  CGGGCAACAG CTGATTGCCC TTCACCGCCT GGCCCTGAGA GAGTTGCAGC
1701  AAGCGGTCCA CGCTGGTTTG CCCCAGCAGG CGAAAATCCT GTTTGATGGT
1751  GGTTAACGGC GGGATATAAC ATGAGCTGTC TTCGGTATCG TCGTATCCCA
1801  CTACCGAGAT ATCCGCACCA ACGCGCAGCC CGGACTCGGT AATGGCGCGC
1851  ATTGCGCCCA GCGCCATCTG ATCGTTGGCA ACCAGCATCG CAGTGGGAAC
1901  GATGCCCTCA TTCAGCATTT GCATGGTTTG TTGAAAACCG GACATGGCAC
1951  TCCAGTCGCC TTCCCGTTCC GCTATCGGCT GAATTTGATT GCGAGTGAGA
2001  TATTTATGCC AGCCAGCCAG ACGCAGACGC GCCGAGACAG AACTTAATGG
2051  GCCCGCTAAC AGCGCGATTT GCTGGTGACC CAATGCGACC AGATGCTCCA
2101  CGCCCAGTCG CGTACCGTCT TCATGGGAGA AAATAATACT GTTGATGGGT
2151  GTCTGGTCAG AGACATCAAG AAATAACGCC GGAACATTAG TGCAGGCAGC
2201  TTCCACAGCA ATGGCATCCT GGTCATCCAG CGGATAGTTA ATGATCAGCC
2251  CACTGACGCG TTGCGCGAGA AGATTGTGCA CCGCCGCTTT ACAGGCTTCG
2301  ACGCCGCTTC GTTCTACCAT CGACACCACC ACGCTGGCAC CCAGTTGATC
2351  GGCGCGAGAT TTAATCGCCG CGACAATTTG CGACGGCGCG TGCAGGGCCA
2401  GACTGGAGGT GGCAACGCCA ATCAGCAACG ACTGTTTGCC CGCCAGTTGT
```

FIG 11 c

```
2451  TGTGCCACGC GGTTGGGAAT GTAATTCAGC TCCGCCATCG CCGCTTCCAC

2501  TTTTTCCCGC GTTTTCGCAG AAACGTGGCT GGCCTGGTTC ACCACGCGGG

2551  AAACGGTCTG ATAAGAGACA CCGGCATACT CTGCGACATC GTATAACGTT

2601  ACTGGTTTCA CATTCACCAC CCTGAATTGA CTCTCTTCCG GGCGCTATCA
            (Met)

2651  TGCCATACCG CGAAAGGTTT TGCACCATTC GATGGTGTCA ACGTAAATGC

                                        SalI
2701  ATGCCGCTTC GCCTTCGCGC GCGAATTGTC GACCCTGTCC CTCCTGTTCA

2751  GCTACTGACG GGGTGGTGCG TAACGGCAAA AGCACCGCCG GACATCAGCG

2801  CTAGCGGAGT GTATACTGGC TTACTATGTT GGCACTGATG AGGGTGTCAG

2851  TGAAGTGCTT CATGTGGCAG GAGAAAAAAG GCTGCACCGG TGCGTCAGCA

2901  GAATATGTGA TACAGGATAT ATTCCGCTTC CTCGCTCACT GACTCGCTAC

2951  GCTCGGTCGT TCGACTGCGG CGAGCGGAAA TGGCTTACGA ACGGGGCGGA

3001  GATTTCCTGG AAGATGCCAG GAAGATACTT AACAGGGAAG TGAGAGGGCC

3051  GCGGCAAAGC CGTTTTTCCA TAGGCTCCGC CCCCCTGACA AGCATCACGA

3101  AATCTGACGC TCAAATCAGT GGTGGCGAAA CCCGACAGGA CTATAAAGAT

3151  ACCAGGCGTT TCCCCTGGCG GCTCCCTCGT GCGCTCTCCT GTTCCTGCCT

3201  TTCGGTTTAC CGGTGTCATT CCGCTGTTAT GGCCGCGTTT GTCTCATTCC

3251  ACGCCTGACA CTCAGTTCCG GGTAGGCAGT TCGCTCCAAG CTGGACTGTA

3301  TGCACGAACC CCCCGTTCAG TCCGACCGCT GCGCCTTATC CGGTAACTAT

3351  CGTCTTGAGT CCAACCCGGA AAGACATGCA AAAGCACCAC TGGCAGCAGC

3401  CACTGGTAAT TGATTTAGAG GAGTTAGTCT TGAAGTCATG CGCCGGTTAA

3451  GGCTAAACTG AAAGGACAAG TTTTGGTGAC TGCGCTCCTC CAAGCCAGTT

3501  ACCTCGGTTC AAAGAGTTGG TAGCTCAGAG AACCTTCGAA AAACCGCCCT

3551  GCAAGGCGGT TTTTTCGTTT TCAGAGCAAG AGATTACGCG CAGACCAAAA

3601  CGATCTCAAG AAGATCATCT TATTAATCAG ATAAAATATT TCTAGATTTC

3651  AGTGCAATTT ATCTCTTCAA ATGTAGCACC TGAAGTCAGC CCCATACGAT

3701  ATAAGTTGTT AATTCTCATG TTTGACAGCT TATCATCGAT
```

FIG 12

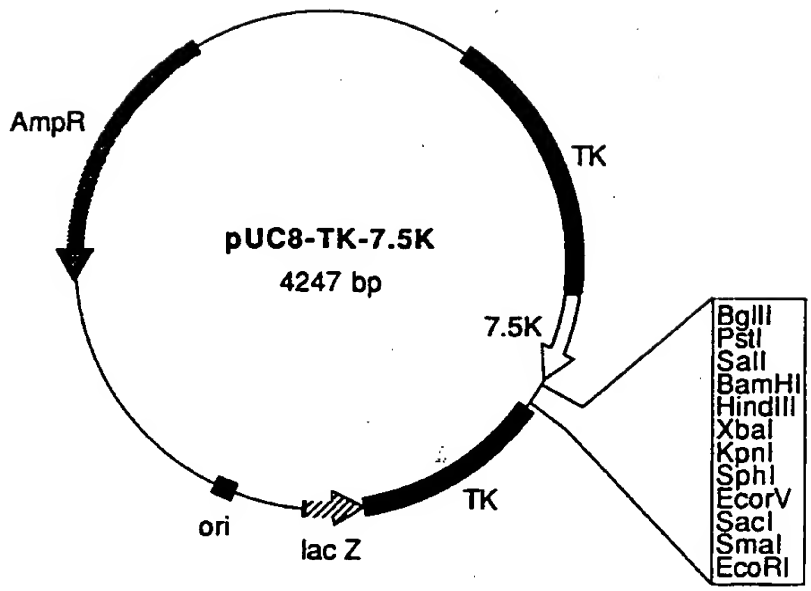

AmpR
TK
pUC8-TK-7.5K
4247 bp
7.5K
TK
ori
lac Z
BglII
PstI
SalI
BamHI
HindIII
XbaI
KpnI
SphI
EcorV
SacI
SmaI
EcoRI

FIG 13

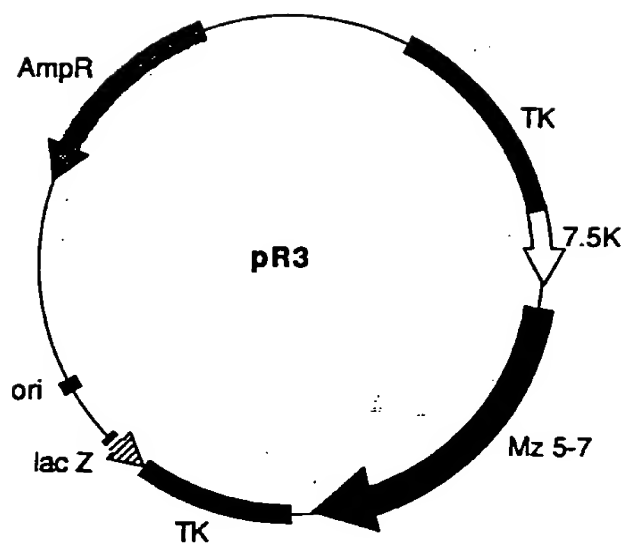


FIG 14 a

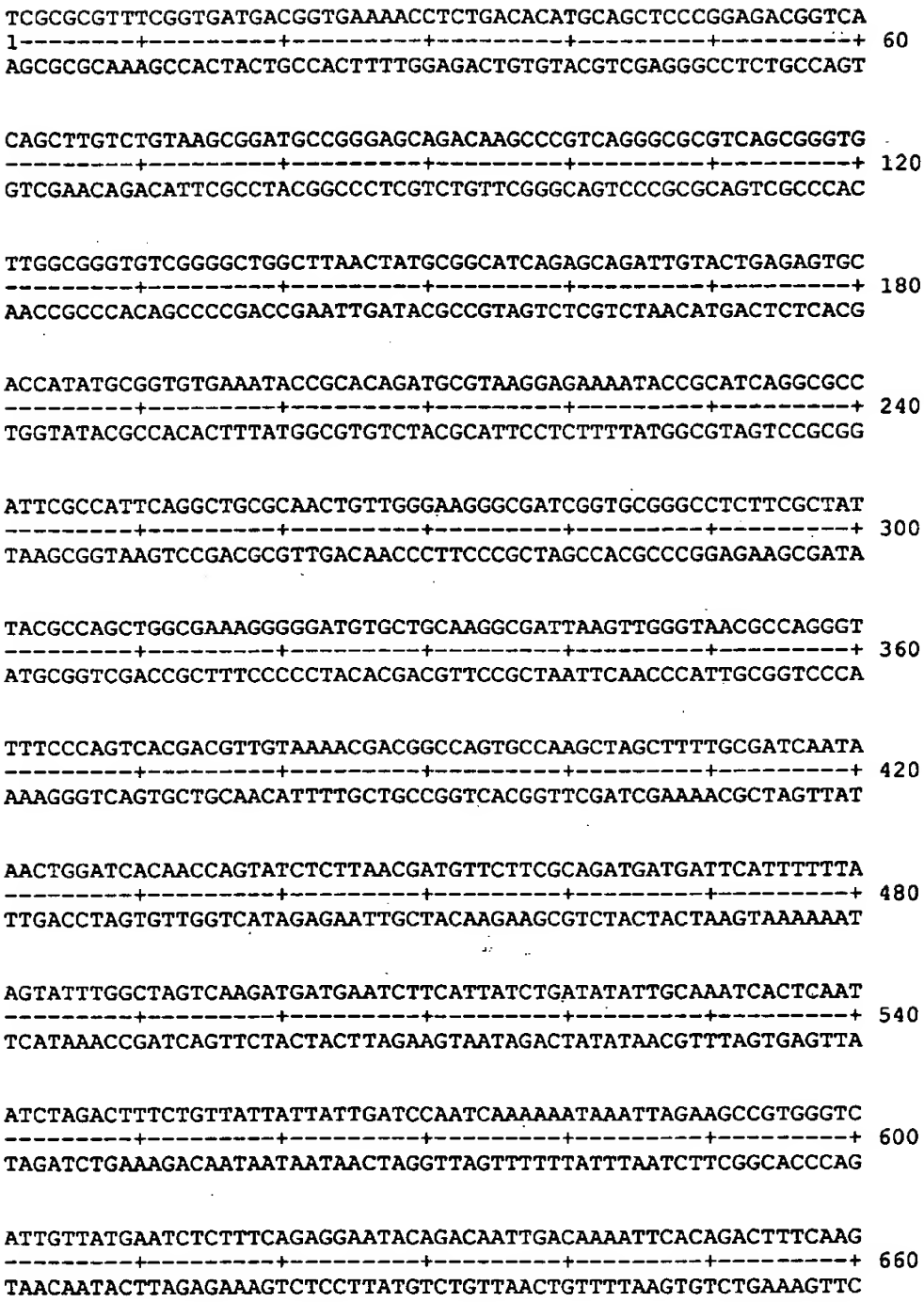

TCGCGCGTTTCGGTGATGACGGTGAAAACCTCTGACACATGCAGCTCCCGGAGACGGTCA
AGCGCGCAAAGCCACTACTGCCACTTTTGGAGACTGTGTACGTCGAGGGCCTCTGCCAGT
60
CAGCTTGTCTGTAAGCGGATGCCGGGAGCAGACAAGCCCGTCAGGGCGCGTCAGCGGGTG
GTCGAACAGACATTCGCCTACGGCCCTCGTCTGTTCGGGCAGTCCCGCGCAGTCGCCCAC
120
TTGGCGGGTGTCGGGGCTGGCTTAACTATGCGGCATCAGAGCAGATTGTACTGAGAGTGC
AACCGCCCACAGCCCCGACCGAATTGATACGCCGTAGTCTCGTCTAACATGACTCTCACG
180
ACCATATGCGGTGTGAAATACCGCACAGATGCGTAAGGAGAAAATACCGCATCAGGCGCC
TGGTATACGCCACACTTTATGGCGTGTCTACGCATTCCTCTTTTATGGCGTAGTCCGCGG
240
ATTCGCCATTCAGGCTGCGCAACTGTTGGGAAGGGCGATCGGTGCGGGCCTCTTCGCTAT
TAAGCGGTAAGTCCGACGCGTTGACAACCCTTCCCGCTAGCCACGCCCGGAGAAGCGATA
300
TACGCCAGCTGGCGAAAGGGGGATGTGCTGCAAGGCGATTAAGTTGGGTAACGCCAGGGT
ATGCGGTCGACCGCTTTCCCCCTACACGACGTTCCGCTAATTCAACCCATTGCGGTCCCA
360
TTTCCCAGTCACGACGTTGTAAAACGACGGCCAGTGCCAAGCTAGCTTTTGCGATCAATA
AAAGGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGATCGAAAACGCTAGTTAT
420
AACTGGATCACAACCAGTATCTCTTAACGATGTTCTTCGCAGATGATGATTCATTTTTTA
TTGACCTAGTGTTGGTCATAGAGAATTGCTACAAGAAGCGTCTACTACTAAGTAAAAAAT
480
AGTATTTGGCTAGTCAAGATGATGAATCTTCATTATCTGATATATTGCAAATCACTCAAT
TCATAAACCGATCAGTTCTACTACTTAGAAGTAATAGACTATATAACGTTTAGTGAGTTA
540
ATCTAGACTTTCTGTTATTATTATTGATCCAATCAAAAAATAAATTAGAAGCCGTGGGTC
TAGATCTGAAAGACAATAATAATAACTAGGTTAGTTTTTTATTTAATCTTCGGCACCCAG
600
ATTGTTATGAATCTCTTTCAGAGGAATACAGACAATTGACAAAATTCACAGACTTTCAAG
TAACAATACTTAGAGAAAGTCTCCTTATGTCTGTTAACTGTTTTAAGTGTCTGAAAGTTC
660

FIG 14 b

```
ATTTTAAAAAACTGTTTAACAAGGTCCCTATTGTTACAGATGGAAGGGTCAAACTTAATA
---------+---------+---------+---------+---------+---------+ 720
TAAAATTTTTTGACAAATTGTTCCAGGGATAACAATGTCTACCTTCCCAGTTTGAATTAT

AAGGATATTTCTTCGACTTTGTGATTAGTTTGATGCGATTCAAAAAAGAATCCTCTCTAG
---------+---------+---------+---------+---------+---------+ 780
TTCCTATAAAGAAGCTGAAACACTAATCAAACTACGCTAAGTTTTTTCTTAGGAGAGATC

CTACCACCGCAATAGATCCTGTTAGATACATAGATCCTCGTCGCAATATCGCATTTTCTA
---------+---------+---------+---------+---------+---------+ 840
GATGGTGGCGTTATCTAGGACAATCTATGTATCTAGGAGCAGCGTTATAGCGTAAAAGAT

ACGTAGTGGATATATTAAAGTCGAATAAAGTGAACAATAATTAATTCTTTATTGTCATCA
---------+---------+---------+---------+---------+---------+ 900
TGCATCACCTATATAATTTCAGCTTATTTCACTTGTTATTAATTAAGAAATAACAGTAGT

TGAACGGCGGACATATTCAGTTGATAATCGGCCCCATGTTTTCAGGTAAAAGTACAGAAT
---------+---------+---------+---------+---------+---------+ 960
ACTTGCCGCCTGTATAAGTCAACTATTAGCCGGGGTACAAAAGTCCATTTTCATGTCTTA

TAATTAGACGAGTTAGACGTTATCAAATAGCTCAATATAAATGCGTGACTATAAAATATT
---------+---------+---------+---------+---------+---------+ 1020
ATTAATCTGCTCAATCTGCAATAGTTTATCGAGTTATATTTACGCACTGATATTTTATAA

CTAACGATAATAGATACGGAAGGGGACTATGGACGCATGATAAGAATAATTTTGAAGCAT
---------+---------+---------+---------+---------+---------+ 1080
GATTGCTATTATCTATGCCTTCCCCTGATACCTGCGTACTATTCTTATTAAAACTTCGTA

TGGAAGCAACTAAACTATGTGATGTCTTGGAATCAATTACAGATTTCTCCGTGATAGGTA
---------+---------+---------+---------+---------+---------+ 1140
ACCTTCGTTGATTTGATACACTACAGAACCTTAGTTAATGTCTAAAGAGGCACTATCCAT

TCGACATCTATATACTATATAGTAATACCAATACTCAAGACTACGAAACTGATACAATCT
---------+---------+---------+---------+---------+---------+ 1200
AGCTGTAGATATATGATATATCATTATGGTTATGAGTTCTGATGCTTTGACTATGTTAGA

CTTATCATGTGGGTAATGTTCTCGATGTCGAATAGCCATATGCCGGTAGTTCGCATATAC
---------+---------+---------+---------+---------+---------+ 1260
GAATAGTACACCCATTACAAGAGCTACAGCTTATCGGTATACGGCCATCAAGCGTATATG

ATAAACTGATCACTAATTCCAAACCCACCCGCTTTTTATAGTAAGTTTTTCACCGATAAA
---------+---------+---------+---------+---------+---------+ 1320
TATTTGACTAGTGATTAAGGTTTGGGTGGGCGAAAAATATCATTCAAAAAGTGGCTATTT
```

FIG 14 c

```
TAATAAATACAATAATTAATTTCTCGTAAAAGTAGAAAATATATTCTAATTTATTGCACG
---------+---------+---------+---------+---------+---------+ 1380
ATTATTTATGTTATTAATTAAAGAGCATTTTCATCTTTTATATAAGATTAAATAACGTGC

GTAAGGAAGTAGAATCATAAAGAACAGTCAGATCGGGAATTCGGCTTTTGCGTCGGAGAT
---------+---------+---------+---------+---------+---------+ 1440
CATTCCTTCATCTTAGTATTTCTTGTCAGTCTAGCCCTTAAGCCGAAAACGCAGCCTCTA

AGTCGTTGTGTGTTTGCGCGATCACCCGCGAACTTCTCTACCAACTGAAAATGGCTAAGT
---------+---------+---------+---------+---------+---------+ 1500
TCAGCAACACACAAACGCGCTAGTGGGCGCTTGAAGAGATGGTTGACTTTTACCGATTCA

                                                  M  A  K  S

CTATGCTTTCTGGAATTGTTTTTGCTGGTCTTGTTGCTGCTGCAGCGGCCAGTTCGGCCA
---------+---------+---------+---------+---------+---------+ 1560
GATACGAAAGACCTTAACAAAAACGACCAGAACAACGACGACGTCGCCGGTCAAGCCGGT

  M  L  S  G  I  V  F  A  G  L  V  A  A  A  A  A  S  S  A  N

ACAGCGCCGCCAACGTCTCCGTTTTGGAGAGTGGGCCCGCTGTGCAGGAAGTGCCAGCGC
---------+---------+---------+---------+---------+---------+ 1620
TGTCGCGGCGGTTGCAGAGGCAAAACCTCTCACCCGGGCGACACGTCCTTCACGGTCGCG

  S  A  A  N  V  S  V  L  E  S  G  P  A  V  Q  E  V  P  A  R

GCACGGTCACAGCTCGCCTGGCGAAGCCTTTGCTGCTTCTTTCTGCTCTTGCTGCGACTT
---------+---------+---------+---------+---------+---------+ 1680
CGTGCCAGTGTCGAGCGGACCGCTTCGGAAACGACGAAGAAAGACGAGAACGACGCTGAA

  T  V  T  A  R  L  A  K  P  L  L  L  L  S  A  L  A  A  T  L

TGGCAGCAGCTTTCCTCGTTTTGCAATGCTTCAACAGCATCTCCAGCAACAACCAGCAAA
---------+---------+---------+---------+---------+---------+ 1740
ACCGTCGTCGAAAGGAGCAAAACGTTACGAAGTTGTgGTAGAGGTCGTTGTTGGTCGTTT

  A  A  A  F  L  V  L  Q  C  F  N  T  I  S  S  N  N  Q  Q  T

CCAGCGTCAGGAGACTGGCCGCCGGAGGTGCATGCGGAGATGAGGAAGATGCAGATGAGG
---------+---------+---------+---------+---------+---------+ 1800
GGTCGCAGTCCTCTGACCGGCGGCCTCCACGTACGCCTCTACTCCTTCTACGTCTACTCC

  S  V  R  R  L  A  A  G  G  A  C  G  D  E  E  D  A  D  E  G

GAACTTCACAGCAGGCCAGCCGGAGGAGGAGAAAACCTGATACCCCTGCAGCAGATAAAT
---------+---------+---------+---------+---------+---------+ 1860
CTTGAAGTGTCGTCCGGTCGGCCTCCTCCTCTTTTGGACTATGGGGACGTCGTCTATTTA

  T  S  Q  Q  A  S  R  R  R  R  K  P  D  T  P  A  A  D  K  Y
```

FIG 14 d

```
ACGATTTTGTTGGCGGAACTCCAGTTTCGGTCACTGAGCCGAATGTTGATGAAGTCCTTA
---------+---------+---------+---------+---------+---------+ 1920
TGCTAAAACAACCGCCTTGAGGTCAAAGCCAGTGACTCGGCTTACAACTACTTCAGGAAT

  D  F  V  G  G  T  P  V  S  V  T  E  P  N  V  D  E  V  L  I

TCCAAATTAGAAATAAACAAATCTTTTTGAAGAACCCATGGACTGGACAAGAAGAACAAG
---------+---------+---------+---------+---------+---------+ 1980
AGGTTTAATCTTTATTTGTTTAGAAAAACTTCTTGGGTACCTGACCTGTTCTTCTTGTTC

  Q  I  R  N  K  Q  I  F  L  K  N  P  W  T  G  Q  E  E  Q  V

TTCTAGTACTGGAACGACAAAGTGAAGAACCCATTCTGATTGTGGCGAGGACAAGACAAA
---------+---------+---------+---------+---------+---------+ 2040
AAGATCATGACCTTGCTGTTTCACTTCTTGGGTAAGACTAACACCGCTCCTGTTCTGTTT

  L  V  L  E  R  Q  S  E  E  P  I  L  I  V  A  R  T  R  Q  T

CACTTGAAGGATATCTTGGTAGTCAAGCTCTTGCACAGGACGGAAAGACTGCTAAAGAAG
---------+---------+---------+---------+---------+---------+ 2100
GTGAACTTCCTATAGAACCATCAGTTCGAGAACGTGTCCTGCCTTTCTGACGATTTCTTC

  L  E  G  Y  L  G  S  Q  A  L  A  Q  D  G  K  T  A  K  E  E

AGAAAGTTGAAGGAGGCAAAACTCACAGAAGATATAAAGTCAAGAGCAGCGACCCAGGAT
---------+---------+---------+---------+---------+---------+ 2160
TCTTTCAACTTCCTCCGTTTTGAGTGTCTTCTATATTTCAGTTCTCGTCGCTGGGTCCTA

  K  V  E  G  G  K  T  H  R  R  Y  K  V  K  S  S  D  P  G  Y

ATGGATTCCCATACACCACGGTGCTCGACGGGGTTCCTGTGGGAACAGACGAAGACGGAT
---------+---------+---------+---------+---------+---------+ 2220
TACCTAAGGGTATGTGGTGCCACGAGCTGCCCCAAGGACACCCTTGTCTGCTTCTGCCTA

  G  F  P  Y  T  T  V  L  D  G  V  P  V  G  T  D  E  D  G  Y

ACGTCGTCGAAGTTCTTATGAAAACCGGACCCCATGGAGGAGTCGACATGATGACTAGCA
---------+---------+---------+---------+---------+---------+ 2280
TGCAGCAGCTTCAAGAATACTTTTGGCCTGGGGTAGCTCCTCAGCTGTACTACTGATCGT

  V  V  E  V  L  M  K  T  G  P  H  G  G  V  D  M  M  T  S  T

CAGCATCACAAGGAAAATTCTGCGGAGTGCTTATGGATGACGGAAAAGGAAACCTAGTCG
---------+---------+---------+---------+---------+---------+ 2340
GTCGTAGTGTTCCTTTTAAGACGCCTCACGAATACCTACTGCCTTTTCCTTTGGATCAGC

  A  S  Q  G  K  F  C  G  V  L  M  D  D  G  K  G  N  L  V  D

ATGGACAAGGGAGAAAAATTACCGCCGTTATCGGCATGCTAACTCAACCGGATACCGAGT
---------+---------+---------+---------+---------+---------+ 2400
TACCTGTTCCCTCTTTTTAATGGCGGCAATAGCCGTACGATTGAGTTGGCCTATGGCTCA

  G  Q  G  R  K  I  T  A  V  I  G  M  L  T  Q  P  D  T  E  F
```

FIG 14 e

```
TTAGAAGCGGACCAGGAGACGACGAGGACGACGAGTGAGTGAGCGGAGTTGGCTTTTGTC
---------+---------+---------+---------+---------+---------+---------+ 2460
AATCTTCGCCTGGTCCTCTGCTGCTCCTGCTGCTCACTCACTCGCCTCAACCGAAAACAG

  R  S  G  P  G  D  D  E  D  D  E  -

CCTGTTGATGCCGTTGCCCACTTTCGCAGCTTGCTTGTTTCCTGGGCTTGCCTGTGCCGC
---------+---------+---------+---------+---------+---------+---------+ 2520
GGACAACTACGGCAACGGGTGAAAGCGTCGAACGAACAAAGGACCCGAACGGACACGGCG

GACATGCGCTTGGCGTTCCGCCTGAGTTCTTTCGGACTGTTTTAACTTTTAATTCATTTT
---------+---------+---------+---------+---------+---------+---------+ 2580
CTGTACGCGAACCGCAAGGCGGACTCAAGAAAGCCTGACAAAATTGAAAATTAAGTAAAA

CTACTGCGGCAAAAAAAAAAAAAAAAAAAAAAAAAAAAACCGAATTCTGTGAGCGTATG
---------+---------+---------+---------+---------+---------+---------+ 2640
GATGACGCCGTTTTTTTTTTTTTTTTTTTTTTTTTTTTGGCTTAAGACACTCGCATAC

GCAAACGAAGGAAAAATAGTTATAGTAGCCGCACTCGATGGGACATTTCAACGTAAACCG
---------+---------+---------+---------+---------+---------+---------+ 2700
CGTTTGCTTCCTTTTTATCAATATCATCGGCGTGAGCTACCCTGTAAAGTTGCATTTGGC

TTTAATAATATTTTGAATCTTATTCCATTATCTGAAATGGTGGTAAAACTAACTGCTGTG
---------+---------+---------+---------+---------+---------+---------+ 2760
AAATTATTATAAAACTTAGAATAAGGTAATAGACTTTACCACCATTTTGATTGACGACAC

TGTATGAAATGCTTTAAGGAGGCTTCCTTTTCTAAACGATTGGGTGAGGAAACCGAGATA
---------+---------+---------+---------+---------+---------+---------+ 2820
ACATACTTTACGAAATTCCTCCGAAGGAAAAGATTTGCTAACCCACTCCTTTGGCTCTAT

GAAATAATAGGAGGTAATGATATGTATCAATCGGTGTGTAGAAAGTGTTACATCGACTCA
---------+---------+---------+---------+---------+---------+---------+ 2880
CTTTATTATCCTCCATTACTATACATAGTTAGCCACACATCTTTCACAATGTAGCTGAGT

TAATATTATATTTTTTATCTAAAAAACTAAAAATAAACATTGATTAAATTTTAATATAAT
---------+---------+---------+---------+---------+---------+---------+ 2940
ATTATAATATAAAAAATAGATTTTTTGATTTTTATTTGTAACTAATTTAAAATTATATTA

ACTTAAAAATGGATGTTGTGTCGTTAGATAAACCGTTTATGTATTTTGAGGAAATTGATA
---------+---------+---------+---------+---------+---------+---------+ 3000
TGAATTTTTACCTACAACACAGCAATCTATTTGGCAAATACATAAAACTCCTTTAACTAT

ATGAGTTAAATTACGAACCAGAAAGTGCAAATGAGGCCGCAAAAAAACTGCCGTATCAAG
---------+---------+---------+---------+---------+---------+---------+ 3060
TACTCAATTTAATGCTTGGTCTTTCACGTTTACTCCGGCGTTTTTTTGACGGCATAGTTC
```

FIG 14 f

```
GACAGTTAAAACTATTACTAGGAGAATTATTTTTTCTTAGTAAGTTACAGCGACACGGTA
---------+---------+---------+---------+---------+---------+ 3120
CTGTCAATTTTGATAATGATCCTCTTAATAAAAAAGAATCATTCAATGTCGCTGTGCCAT


TATTAGATGGTGCCACCGTAGTGTATATAGGATCTGCTCCCCGTAATCATGGTCATAGCT
---------+---------+---------+---------+---------+---------+ 3180
ATAATCTACCACGGTGGCATCACATATATCCTAGACGAGGGGCATTAGTACCAGTATCGA


GTTTCCTGTGTGAAATTGTTATCCGCTCACAATTCCACACAACATACGAGCCGGAAGCAT
---------+---------+---------+---------+---------+---------+ 3240
CAAAGGACACACTTTAACAATAGGCGAGTGTTAAGGTGTGTTGTATGCTCGGCCTTCGTA


AAAGTGTAAAGCCTGGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTC
---------+---------+---------+---------+---------+---------+ 3300
TTTCACATTTCGGACCCCACGGATTACTCACTCGATTGAGTGTAATTAACGCAACGCGAG


ACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGGCCAACG
---------+---------+---------+---------+---------+---------+ 3360
TGACGGGCGAAAGGTCAGCCCTTTGGACAGCACGGTCGACGTAATTACTTAGCCGGTTGC


CGCGGGGAGAGGCGGTTTGCGTATTGGGCGCTCTTCCGCTTCCTCGCTCACTGACTCGCT
---------+---------+---------+---------+---------+---------+ 3420
GCGCCCCTCTCCGCCAAACGCATAACCCGCGAGAAGGCGAAGGAGCGAGTGACTGAGCGA


GCGCTCGGTCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTT
---------+---------+---------+---------+---------+---------+ 3480
CGCGAGCCAGCAAGCCGACGCCGCTCGCCATAGTCGAGTGAGTTTCCGCCATTATGCCAA


ATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGC
---------+---------+---------+---------+---------+---------+ 3540
TAGGTGTCTTAGTCCCCTATTGCGTCCTTTCTTGTACACTCGTTTTCCGGTCGTTTTCCG


CAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGA
---------+---------+---------+---------+---------+---------+ 3600
GTCCTTGGCATTTTTCCGGCGCAACGACCGCAAAAAGGTATCCGAGGCGGGGGGACTGCT


GCATCACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATA
---------+---------+---------+---------+---------+---------+ 3660
CGTAGTGTTTTTAGCTGCGAGTTCAGTCTCCACCGCTTTGGGCTGTCCTGATATTTCTAT


CCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTAC
---------+---------+---------+---------+---------+---------+ 3720
GGTCCGCAAAGGGGGACCTTCGAGGGAGCACGCGAGAGGACAAGGCTGGGACGGCGAATG
```

FIG 14 g

```
CGGATACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCAATGCTCACGCTG
---------+---------+---------+---------+---------+---------+ 3780
GCCTATGGACAGGCGGAAAGAGGGAAGCCCTTCGCACCGCGAAAGAGTTACGAGTGCGAC

TAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCC
---------+---------+---------+---------+---------+---------+ 3840
ATCCATAGAGTCAAGCCACATCCAGCAAGCGAGGTTCGACCCGACACACGTGCTTGGGGG

CGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAG
---------+---------+---------+---------+---------+---------+ 3900
GCAAGTCGGGCTGGCGACGCGGAATAGGCCATTGATAGCAGAACTCAGGTTGGGCCATTC

ACACGACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGT
---------+---------+---------+---------+---------+---------+ 3960
TGTGCTGAATAGCGGTGACCGTCGTCGGTGACCATTGTCCTAATCGTCTCGCTCCATACA

AGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGT
---------+---------+---------+---------+---------+---------+ 4020
TCCGCCACGATGTCTCAAGAACTTCACCACCGGATTGATGCCGATGTGATCTTCCTGTCA

ATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTG
---------+---------+---------+---------+---------+---------+ 4080
TAAACCATAGACGCGAGACGACTTCGGTCAATGGAAGCCTTTTTCTCAACCATCGAGAAC

ATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTAC
---------+---------+---------+---------+---------+---------+ 4140
TAGGCCGTTTGTTTGGTGGCGACCATCGCCACCAAAAAAACAAACGTTCGTCGTCTAATG

GCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCA
---------+---------+---------+---------+---------+---------+ 4200
CGCGTCTTTTTTTCCTAGAGTTCTTCTAGGAAACTAGAAAAGATGCCCCAGACTGCGAGT

GTGGAACGAAAACTCACGTTAAGGGATTTTGGTCATGAGATTATCAAAAAGGATCTTCAC
---------+---------+---------+---------+---------+---------+ 4260
CACCTTGCTTTTGAGTGCAATTCCCTAAAACCAGTACTCTAATAGTTTTTCCTAGAAGTG

CTAGATCCTTTTAAATTAAAAATGAAGTTTTAAATCAATCTAAAGTATATATGAGTAAAC
---------+---------+---------+---------+---------+---------+ 4320
GATCTAGGAAAATTTAATTTTTACTTCAAAATTTAGTTAGATTTCATATATACTCATTTG

TTGGTCTGACAGTTACCAATGCTTAATCAGTGAGGCACCTATCTCAGCGATCTGTCTATT
---------+---------+---------+---------+---------+---------+ 4380
AACCAGACTGTCAATGGTTACGAATTAGTCACTCCGTGGATAGAGTCGCTAGACAGATAA
```

FIG 14 h

```
TCGTTCATCCATAGTTGCCTGACTCCCCGTCGTGTAGATAACTACGATACGGGAGGGCTT
---------+---------+---------+---------+---------+---------+ 4440
AGCAAGTAGGTATCAACGGACTGAGGGGCAGCACATCTATTGATGCTATGCCCTCCCGAA

ACCATCTGGCCCCAGTGCTGCAATGATACCGCGAGACCCACGCTCACCGGCTCCAGATTT
---------+---------+---------+---------+---------+---------+ 4500
TGGTAGACCGGGGTCACGACGTTACTATGGCGCTCTGGGTGCGAGTGGCCGAGGTCTAAA

ATCAGCAATAAACCAGCCAGCCGGAAGGGCCGAGCGCAGAAGTGGTCCTGCAACTTTATC
---------+---------+---------+---------+---------+---------+ 4560
TAGTCGTTATTTGGTCGGTCGGCCTTCCCGGCTCGCGTCTTCACCAGGACGTTGAAATAG

CGCCTCCATCCAGTCTATTAATTGTTGCCGGGAAGCTAGAGTAAGTAGTTCGCCAGTTAA
---------+---------+---------+---------+---------+---------+ 4620
GCGGAGGTAGGTCAGATAATTAACAACGGCCCTTCGATCTCATTCATCAAGCGGTCAATT

TAGTTTGCGCAACGTTGTTGCCATTGCTACAGGCATCGTGGTGTCACGCTCGTCGTTTGG
---------+---------+---------+---------+---------+---------+ 4680
ATCAAACGCGTTGCAACAACGGTAACGATGTCCGTAGCACCACAGTGCGAGCAGCAAACC

TATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTT
---------+---------+---------+---------+---------+---------+ 4740
ATACCGAAGTAAGTCGAGGCCAAGGGTTGCTAGTTCCGCTCAATGTACTAGGGGGTACAA

GTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGC
---------+---------+---------+---------+---------+---------+ 4800
CACGTTTTTTCGCCAATCGAGGAAGCCAGGAGGCTAGCAACAGTCTTCATTCAACCGGCG

AGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGT
---------+---------+---------+---------+---------+---------+ 4860
TCACAATAGTGAGTACCAATACCGTCGTGACGTATTAAGAGAATGACAGTACGGTAGGCA

AAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCG
---------+---------+---------+---------+---------+---------+ 4920
TTCTACGAAAAGACACTGACCACTCATGAGTTGGTTCAGTAAGACTCTTATCACATACGC

GCGACCGAGTTGCTCTTGCCCGGCGTCAATACGGGATAATACCGCGCCACATAGCAGAAC
---------+---------+---------+---------+---------+---------+ 4980
CGCTGGCTCAACGAGAACGGGCCGCAGTTATGCCCTATTATGGCGCGGTGTATCGTCTTG

TTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACC
---------+---------+---------+---------+---------+---------+ 5040
AAATTTTCACGAGTAGTAACCTTTTGCAAGAAGCCCCGCTTTTGAGAGTTCCTAGAATGG
```

FIG 14 i

```
GCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
---------+---------+---------+---------+---------+---------+ 5100
CGACAACTCTAGGTCAAGCTACATTGGGTGAGCACGTGGGTTGACTAGAAGTCGTAGAAA

TACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGG
---------+---------+---------+---------+---------+---------+ 5160
ATGAAAGTGGTCGCAAAGACCCACTCGTTTTTGTCCTTCCGTTTTACGGCGTTTTTTCCC

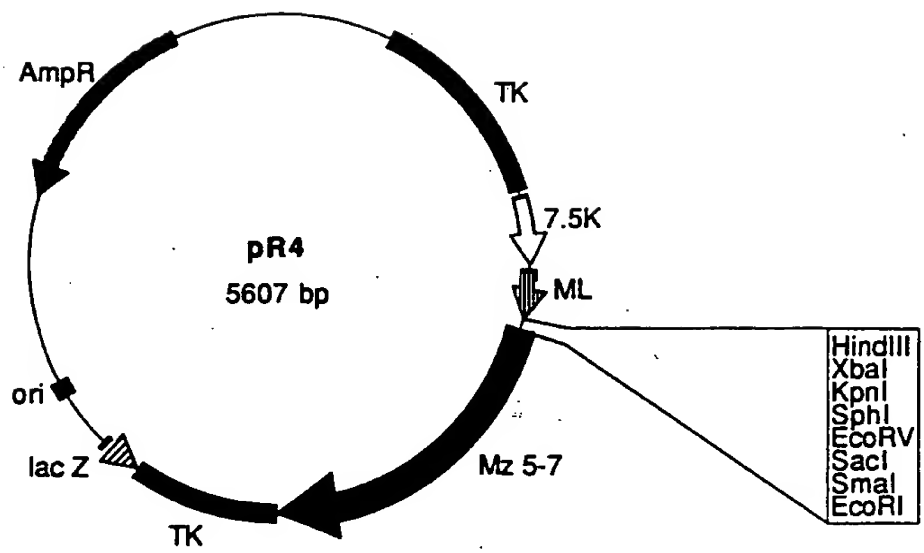
AATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAG
---------+---------+---------+---------+---------+---------+ 5220
TTATTCCCGCTGTGCCTTTACAACTTATGAGTATGAGAAGGAAAAAGTTATAATAACTTC

CATTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAA
---------+---------+---------+---------+---------+---------+ 5280
GTAAATAGTCCCAATAACAGAGTACTCGCCTATGTATAAACTTACATAAATCTTTTTATT

ACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCAT
---------+---------+---------+---------+---------+---------+ 5340
TGTTTATCCCCAAGGCGCGTGTAAAGGGGCTTTTCACGGTGGACTGCAGATTCTTTGGTA

TATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC
---------+---------+---------+---------+---------+--- 5393
ATAATAGTACTGTAATTGGATATTTTTATCCGCATAGTGCTCCGGGAAAGCAG
```

FIG 15


AmpR
TK
7.5K
ML
pR4
5607 bp
ori
lac Z
TK
Mz 5-7
HindIII
XbaI
KpnI
SphI
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SmaI
EcoRI